The Influence of Water on the Glucose Affinity of Hexokinase.

by

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A Thesis
submitted to the Department of Biological Sciences
in partial fulfillment of the requirements for
the degree of
Master of Science

June 1995

Brock University
St. Catharines, Ontario

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Abstract.

In the present thesis, the role of hydration during the glucose induced conformational change of hexokinase is investigated. This is accomplished by applying the osmotic stress technique. The osmotic stress technique is founded on varying of the activity of water in a system in order to determine it's effects. This is accomplished by adding inert solute molecules that are excluded from the system under study. The solute molecules used within the present investigation are Polyethylene glycols (PEGs). PEGs aid in the removal of water from hexokinase by exerting osmotic pressure. The osmotic pressures of the PEG solutions are also measured with both vapour pressure osmometry and secondary osmometry with phospholipids. An interesting discovery is made in that the osmotic pressures of PEG and co-solute solutions are non-additive. This indicates that PEG concentrates co-solutes in solution by making a certain proportion of the water inaccessible. Glucose binding was measured fluorometrically and the glucose equilibrium dissociation constant (GEDC) of hexokinase is measured in solutions containing the different MW PEGs. Changes in the sensitivity of the glucose affinity with osmotic pressure allows the calculation of the change in the numbers of polymer-inaccessible water molecules upon the binding of glucose to hexokinase ΔN_w. It was determined the ΔN_w decreases with increases in osmotic pressure in the presence of all MW PEGs. ΔN_w decreases from values between 45-290 water molecules at low pressure to approximately 15 at high pressure. There is also a molecular weight dependence observed. There are large decreases in ΔN_w with osmotic pressure in the presence of PEGs above MW 1000. However, below MW 1500 changes in ΔN_w with osmotic pressure are relatively small. These findings are interpreted with respect to two possible mechanisms involving changes in the conformation of hexokinase under osmotic pressure and the access of the PEG molecules to water surrounding hexokinase.

Acknowledgments.

It is with great respect and admiration that I thank Dr. R. Peter Rand for his support and consideration. Although times were often trying, his support and understanding have allowed the completion of this work. He has provided me with insight into the character of a great researcher and individual. Above all, he has shown me that the most exciting part of research is not necessarily in confirming ones predictions, but that there is thrill in interpreting unpredicted results. I wish to express my great respect and admiration of Mrs. Nola Fuller. She is an individual with extraordinary qualities, such as youthful enthusiasm, and strength of character, that allow her to cope with results that are predictably unpredictable. Her experience and teaching gave me the basis by which to start my project. Her input during discussions has been invaluable. I would also like to express my thanks to, and great admiration of Dr. V. Adrian Parsegian. He has shown me that it is possible to be both brilliant in ones career and character. I would like to thank Dr. P. Nichols for the use of his lab and also for sharing his unique perspectives from time to time. I also wish to thank Dr. Jack Kornblatt for his expertise in fluorescence spectroscopy that showed me avenues in order to fine tune my experimental protocols and Dr. Bruce for his insightful input. And a special thanks to Mr. Axel Sinner who has shown me a rare combination of raw determination and self-control that has been an important lesson in my life. To our secretaries Shelly and Janet, and store keepers, Barbara and Marilyn I extend sincere thanks for both supportive attitudes and expertise in dealing with University bureaucracy that would otherwise grind all work to a halt. I would also like to express my thanks to Mary Maj. She is an individual with unparalleled clarity of thought who will never lose the sight of her overall objectives even in the presence of overwhelming details.

This thesis is dedicated to the memories of my Sister Mary Jane Reid, my loving Mother Patricia J. Reid, and to my Uncle Ronald C. Evans. Just knowing that they were always in my corner has always been my greatest source of strength and pride. To my Father John A. Reid, I wish to thank for his love, patience, and understanding in the face of very trying times.

Introduction.

Contact between surfaces mediates many of the cellular processes that are the foundations of life. Some important examples are membrane fusion during exocytosis, and the contact between enzyme and ligand during enzymatic catalysis. Knowledge of how these processes occur requires an accurate description of the forces that mediate contact. Detailed studies have allowed the elucidation of several key forces.

Research over several decades has discovered and described several forces that exist between surfaces. These are: steric forces, electrostatic repulsive forces, van der Waals forces of attraction, hydration repulsive forces, and hydration attractive forces (Rand & Parsegian, 1992). These forces act in concert, and have been measured to characterize their relative contribution.

The effect of hydration within a system can be investigated with the osmotic stress technique. This technique alters the activity of water within systems that are in thermodynamic equilibrium in order to study the effects of hydration. LeNeveu et al. (1976) pioneered the application of this technique to the neutral phospholipid, lecithin, in order to characterize the repulsive forces that affect bilayer association in an aqueous system. More recently, this technique has been applied to more complex systems, such as ion channels (Rayner et al., 1992; Zimmerberg & Parsegian, 1986), and enzyme systems (Kornblatt & Bon Hoa, 1990 Rand et al., 1993).

The formation and maintenance of native protein conformations are primarily driven by the hydrophobic interactions between water and amino acid residues (Dill, 1990; Tanford, 1978). The contribution of hydration to conformational changes in the structures of proteins can

be measured with the osmotic stress technique. Recently, the enzyme hexokinase has been so studied (Rand et al., 1993).

Hexokinase is a particularly good enzyme for osmotic stress measurements because of several characteristics: hexokinase is active as a single polypeptide chain, eliminating possible complications due to quaternary structure (Bennett & Steitz, 1978); it undergoes a substrate induced conformational change that is fluorometrically observable (Hoggett & Kellett, 1976; 1992; Zewe et al., 1964); and the crystal structures of both open and closed forms are known (Bennett & Steitz, 1978).

The present investigation is an extension of the earlier work performed by Rand et al. (1993). The work has focused on developing a procedure to more accurately measure the relationships between osmotic pressure and the affinity of hexokinase for its substrate glucose. Different molecular weight (MW) polyethylene glycol (PEG) polymers have been used to measure the relationship between osmotic pressure and the change in volume of polymer-inaccessible water upon the binding of glucose to hexokinase.

Literature Review.

Hexokinase.

Glycolysis is the most completely characterized metabolic pathway in biology (Voet & Voet, 1990), and its first enzymatic reaction is mediated by hexokinase (Berger et al., 1946; Colowick & Kalckar, 1943; Kunitz & McDonald, 1946; Meyerhoff, 1927). Hexokinase exists as various isozymes found in a wide variety of species (Baijal & Wilson, 1992; Ganedo et al., 1977; Kriegel et al., 1994; Walsh et al., 1991). In mammals, these isozymes can be associated with a voltage dependent anion channel located in mitochondrial membranes (Baijal & Wilson, 1992; Nakashima et al., 1986; Stoffel et al., 1992). Hexokinase catalyses the phosphorylation of hexose sugars according to the following reaction (Colowick, 1973; Voet & Voet, 1990):

HK-ATP + hexose<=>HK*-ATP-hexose<=>HK + hexose-6-phosphate + ADP

where, HK and HK* are the opened and closed conformations of the enzyme, respectively (Figs. 1 and 2)(Berger et al., 1946; Kunitz & McDonald, 1946). Further discussion of hexokinase is restricted to those forms found within the yeast, *Saccharomyces cerevisiae*. Within wild type yeast, hexokinase mediates hexose phosphorylation with two different isozymes coded for on two different structural genes (Colowick, 1973). These isozymes are: Hexokinase 1 (PI, gene HXK1)(Kopetzki et al., 1985), and Hexokinase 2 (PII, gene HXK2)(Fröhlich et al., 1985; Kopetzki & Entian, 1985). Glucokinase is another hexose phosphorlyating enzyme that is specific for glucose and is encoded on the gene GLU (Albig & Entian, 1988). At least one of these genes is necessary for the growth of yeast in the presence of glucose (Gancedo et al., 1977). HXK 1 and 2 share 76% base pair homology (Kopetzki et al., 1985), while HXK 1 and 2 share only 28% base pair homology with

Figure 1. A computer generated representation of the conformation of hexokinase that is not bound to glucose. The yellow structure is a space filling model of hexokinase. The red structure is a space filling model of glucose. All structures are shown surrounded by water. The models were generated on a Silicone Graphics Extreme computer using the program package Quanta (Molecular Simulations, Waltham, MA).

Water
17

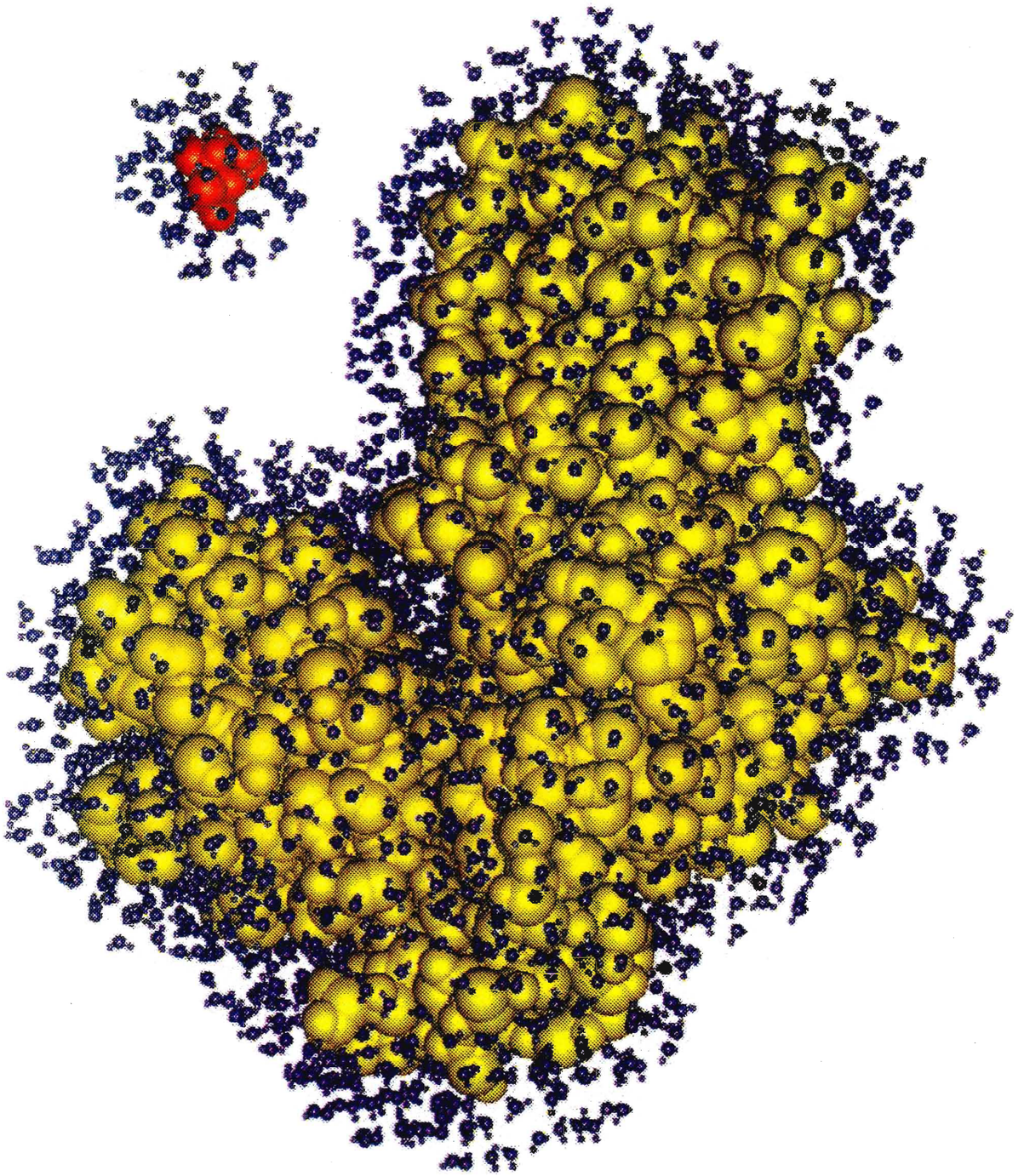
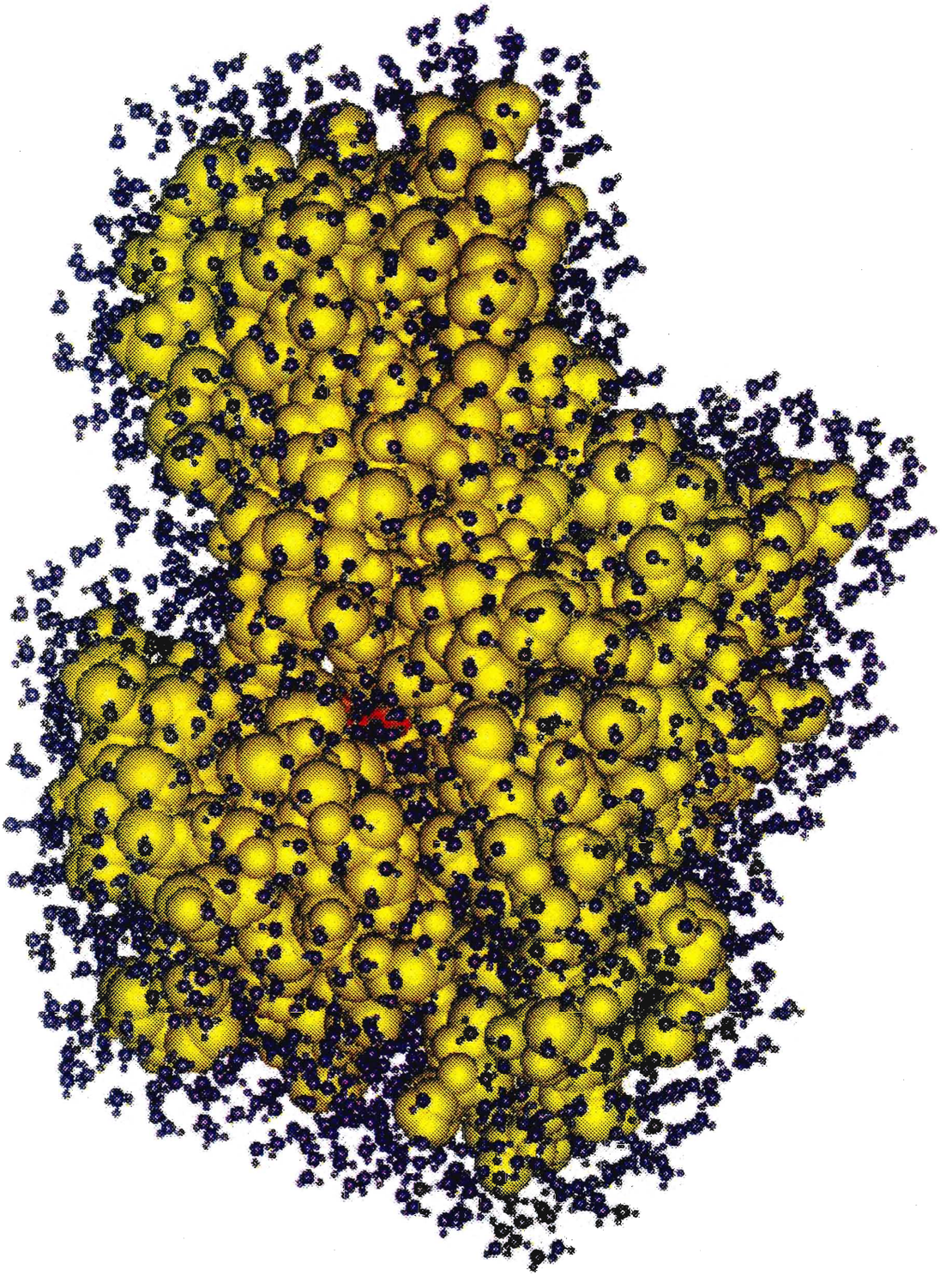


Figure 2. A computer generated representation of the glucose bound conformation of hexokinase. The yellow structure is a space filling model of hexokinase. The red structure is a space filling model of glucose. All structures are shown surrounded by water. The models were generated on a Silicone Graphics Extreme computer using the program package Quanta (Molecular Simulations, Waltham, MA).

Water
19



GLU (Albig & Entian, 1988). Interestingly, the ATP binding domains of the hexokinase isozymes have been shown to be part of a super-family of structurally related proteins including heat shock 70 proteins (Flaherty et al., 1990).

The low resolution crystal structures of hexokinase 1 and 2 have been published in a series of papers by Steitz et al. (Anderson et al., 1978; Bennett, & Steitz, 1978, 1980; Fletterick et al., 1975; Steitz et al., 1976, 1981). The hexokinase isozymes are 53,000 Da. proteins, composed of single polypeptide chains, that can exist as catalytically active monomers or dimers (Bennett & Steitz, 1978). Studies have shown that hexokinase 1 and 2 have identical catalytic activity (Kaji et al., 1961).

Hexokinases catalyze the phosphorylation of many hexose sugars, such as D-glucose, D-fructose, and D-mannose (Berger et al., 1946). These isozymes require magnesium for activity (Berger et al., 1946), and are activated by citrate, phosphate, and 3-phosphoglycerate (Kosow & Rose, 1971). Hexokinases are inhibited by ADP and GDP (Womack et al., 1973). More recently, genetic studies have discovered that hexokinases are regulated by the production of trehalose-6-phosphate, a metabolite that exists within yeast at physiologically significant concentrations (Blazquez et al., 1993). Many of the characteristics of this enzyme's regulation and substrate specificity result from how these ligands effect the conformational change that hexokinase undergoes during its catalytic cycle.

The conformational change that occurs upon hexose binding to hexokinase isozymes involves the rotation of one lobe of the enzyme 12°, or 8 Å, with respect to the other (Bennett & Steitz, 1978). This is a classic example of the substrate-induced fit mechanism of enzyme

catalysis (Koshland et al., 1966), and is essential to the catalytic cycle of the enzyme (Anderson et al., 1978). Interestingly, the two lobes of the isozymes are hypothesized to be created as a result of a gene duplication that has left the lobes symmetrically matched for closure (McLachlan, 1979).

The closure of the cleft during catalysis has been shown to be essential for hexokinase 2 catalysis by investigating the mechanism of competitive inhibition by *o*-toluoylglucosamine (OTG) (Anderson et al., 1978). Low resolution X-ray crystallography indicates that both glucose and OTG dock in precisely the same orientation in the open conformation of hexokinase 2 (Anderson et al., 1978; Fletterick et al., 1975). It has also been shown that the positions of the reactive 6-hydroxyl groups in both glucose and OTG, relative to hexokinase 2, are identical (Anderson et al., 1978; Fletterick et al., 1975). However, OTG will not induce the conformational change in hexokinase 2 (Anderson et al., 1978; Fletterick et al., 1975). Anderson et al. (1978) propose that because OTG contains a bulky toluyl group positioned within the cleft of the enzyme, this makes it sterically impossible for cleft closure. This provides supportive evidence that cleft closure is necessary for catalysis of hexokinase 2, but does not give any indication as to what part of the catalytic cycle requires it.

The elimination of water from the active site of hexokinase is also important. Earlier investigations have shown that in the absence of substrate, a mixture of the hexokinase isozymes exhibits low level ATPase activity (Kaji et al., 1961; Kaji & Colowick, 1965). Dela Fuente et al. (1970) subsequently used substrates lacking the reactive 6-hydroxyl group, so that they could not be phosphorylated by the kinase. Their results indicated that after cleft closure, the K_m for ATP in the

ATPase reaction rivaled that of the kinase reaction. This has led Bennett and Steitz (1980) to hypothesize that the conformational change dependent extrusion of water from the active site is required for efficient kinase activity.

Hexokinase plays an important role in the carbon catabolism in the yeast *S. cerevisiae* (Fernandez et al., 1986, 1987, 1988). Exposure of hexokinase 2 to D-xylose (which lacks the 6-hydroxyl group), causes non-competitive inhibition of the isozyme, as well as autophosphorylation of the protein (Fernandez et al., 1988; Fröhlich et al., 1985). By some as yet unknown mechanism, this autophosphorylation induces derepression of maltase and invertase synthesis (Fernandez et al., 1986). This finding shows that hexokinase is not only involved in hexose sugar metabolism, but also has a role in controlling other aspects of cellular metabolism.

The hexokinase isozymes exist as dimers at neutral pH and low ionic strength conditions, but dissociate at high pH and ionic strength (Kenkare & Colowick, 1965; Hoggett & Kellett, 1976; Wasylewski et al., 1985). The monomeric form of hexokinase has also been measured to be favored in the presence of glucose (Derechin et al., 1972; Feldman & Kramp, 1978; Hoggett & Kellett, 1976, 1992). The binding of glucose to the monomeric forms of hexokinase is stronger than to the dimers (Hoggett & Kellett, 1976, 1992).

A small segment of 11 amino acids mediates the dimerization of both hexokinase 1 and hexokinase 2 (Schulze & Colowick, 1969). These reasearchers have discovered that proteolytic digestion of monomeric hexokinases will remove these amino acids and convert them into the catalytically equivalent S-form isozymes. These isozymes lack the ability to dimerize (Ruan & Weber, 1988; Schulze & Colowick, 1969).

The regions of amino acids responsible for dimerization were later isolated, sequenced, and shown to be highly basic (Fröhlich et al., 1985; Schmidt & Colowick, 1973).

Fluorescence Spectroscopy of Hexokinase.

Fluorescence is the process that occurs when a substance (fluorophore) absorbs light at a particular wavelength and then emits it at a different wavelength (Lakowicz, 1983). Without light absorption, fluorophores will have an intrinsic amount of energy due to the vibration and rotation of its molecules (Lakowicz, 1983). Light absorption elevates the fluorophore to higher electronic states (Lakowicz, 1983). Since light energy is composed of discrete energy units (quanta), then fluorophores are elevated to discrete electronic states (Lakowicz, 1983). This does not mean that all the fluorophores will attain the same excited state because the process is influenced by the initial amount of rotational and vibrational energy of the fluorophore (Demchenko, 1992). Thus, there are many different states that a molecule may jump to when it is excited by a quantum of light.

Once the molecule is excited, it may lose its energy through emission, collision, or pass it on to a lower vibrational state of equivalent energy (internal conversion) (Lakowicz, 1983). The usefulness of fluorescence spectroscopy is founded on the fact that fluorophores have stable properties (Lakowicz, 1983). By observing changes in these properties resulting from experimental conditions, one may gather information about the environment that surrounds the fluorophore (Altekar, 1977 a,b; Lakowicz, 1983; Lakowicz & Weber, 1973). Of great interest to biology is the study of certain amino acid fluorophores in proteins.

Tryptophan is a very common amino acid (Beechum & Brand, 1985), which accounts for approximately 90% of the fluorescence in proteins (Lakowicz, 1983). It absorbs light maximally at approximately 280 nm and shows fluorescent emissions between 320-350 nm (Lakowicz, 1983). Since this amino acid is an integral part of many proteins, tryptophan fluorescence studies are used to gather information about the structure and shape changes of proteins.

DNA sequencing has shown that there are four tryptophan residues contained within the two hexokinase isozymes (Fröhlich et al., 1985; Kopetzki et al., 1985; Kopetzki & Entian, 1985). Peptide mapping (Schmidt & Colowick, 1973), and fluorescence signature studies (Feldman & Kramp, 1978), have also shown four tryptophan residues to exist in hexokinase 2. Fluorometric investigation into the tryptophan distribution in both hexokinase isozymes show that there are two surface residues, of which the first is highly solvent accessible, and the second has limited solvent accessibility (probably in a surface crevice)(Norton & Feldman, 1980). The third residue is buried in the hydrophobic interiors of the isozymes, and the fourth residue is contained within the clefts of the isozymes (Norton & Feldman, 1980). The fourth tryptophan residue is responsible for the substrate induced quenching that is characteristic of yeast hexokinase isozymes (Norton & Feldman, 1980).

The decrease in the intrinsic fluorescence of hexokinases upon glucose binding is not due to the direct interaction of glucose with the fluorophores (Feldman & Kramp, 1978). The fact that the quenching occurs at very low glucose concentrations shows that a strictly collisional quenching mechanism is unlikely (Feldman & Kramp, 1978). The quenching is likely due to changes in the environment around the

fluorophores as a result of the conformational change that occurs when glucose binds (Feldman & Kramp, 1978). These changes in environment are due to interactions between the tryptophan residue and other amino acid side chains (Feldman & Kramp, 1978). In this report, fluorescence spectroscopy is used to measure glucose binding to a mixture of hexokinase isozymes.

From the decrease in the intrinsic fluorescence of hexokinase, one can measure its Glucose Equilibrium Dissociation Constant (GEDC). The GEDC is the concentration at which half the enzymes are bound to glucose. This assay allows the measurement of glucose binding in a simple, single substrate-enzyme system, where one glucose molecule will bind to one active site on hexokinase. This is described by the following reaction:

$$[HK^{*} \bullet Glucose] \underset{K_1}{\overset{K_2}{\Leftrightarrow}} [HK] + [Glucose] \qquad (1)$$

where HK* and HK are considered to be the closed and open conformations respectively. The GEDC is equal to: $K_d = K_2/K_1 =$ [HK][Glucose]/[HK*Glucose]. In order to measure the fraction of hexokinase bound to glucose ([HK*Glucose]) one can titrate the decrease in the intrinsic fluorescence with respect to the maximal fluorescence change at saturating glucose concentrations ({[HK]+[HK*Glucose]}): This is described by the following equation:

$$\frac{\Delta F}{\Delta F_{\max}} = \frac{[HK^{*} \bullet Glucose]}{\{[HK] + [HK^{*} \bullet Glucose]\}} \qquad (2)$$

This relationship allows one to measure glucose binding to hexokinase by following the decreases in intrinsic fluorescence upon the addition of glucose. The GEDC can then be determined by the double reciprocal plots (Hoggett & Kellett, 1976; Zewe et al., 1964), or by a

non-linear computerized fitting technique on a Macintosh LC 475 (Multifit: version 2). Subsequently within this report, changes in the GEDC will be studied as a function of applied osmotic pressure.

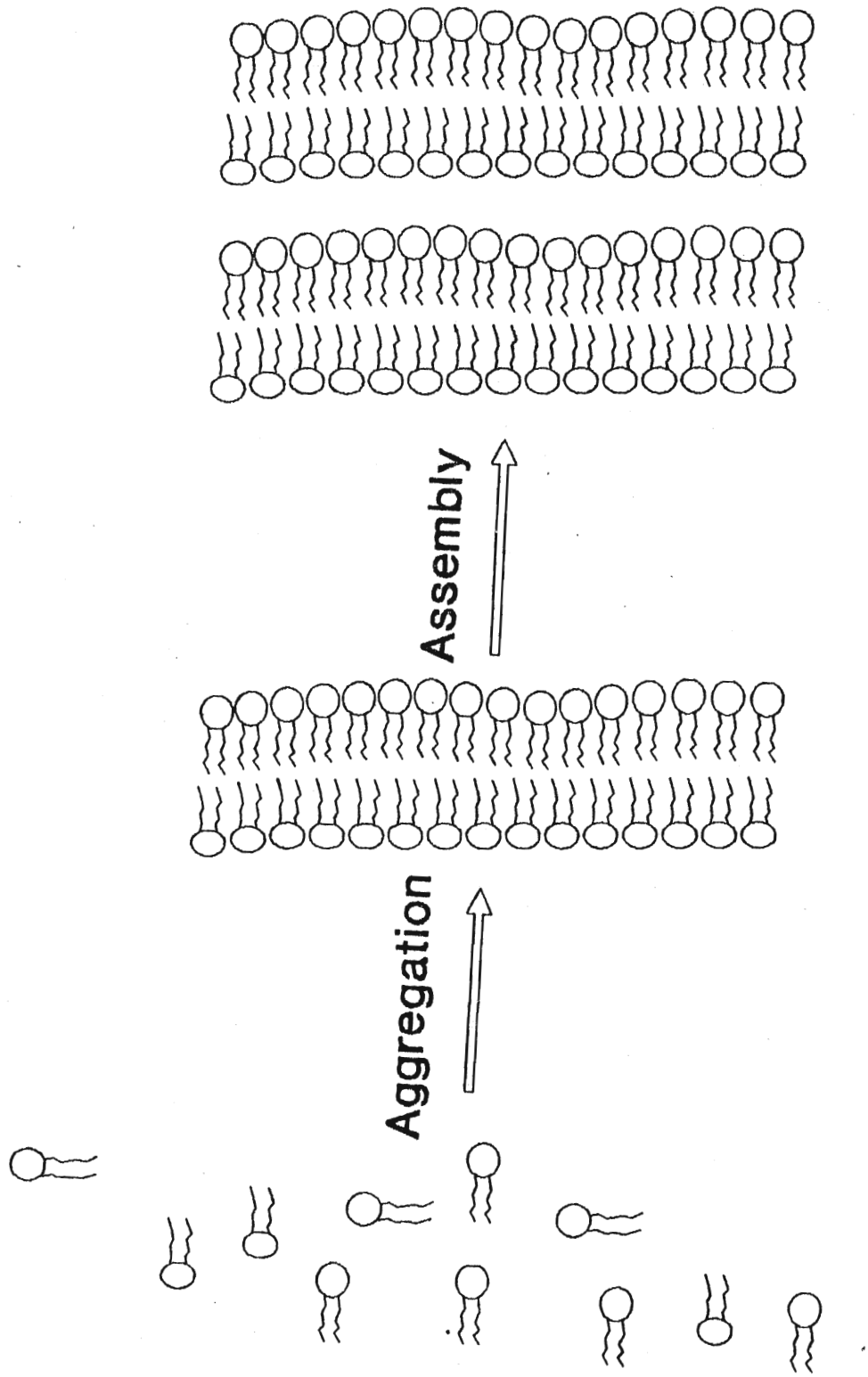
Investigating the Forces in Aqueous Systems that Affect the Association of Surfaces.

Model systems of simple surfaces, such as atomically smooth mica, or phospholipid structures, have been used to measure the forces important to the interactions between surfaces. Phospholipids are well suited to force studies because they spontaneously adopt characteristic structures when placed in solutions with controlled lipid and aqueous components. A well characterized structure that phospholipids often adopt, the lamellar phase (Fig. 3), consists of equidistant parallel stacks of lipid bilayers (Luzzati, 1968; Rand, 1981). The study of this phase has been important in measuring and characterizing the forces that exist between bilayer surfaces (Leiken et al., 1993; Rand, 1981; Rand & Parsegian, 1992). The observed forces are: van der Waals forces of attraction, electrostatic repulsion forces, and hydration forces (Leiken et al., 1993; Rand, 1981; Rand & Parsegian, 1992). The origins of these forces are described below.

Forces that Mediate the Association of Phospholipid Bilayers.

Electrostatic repulsion forces are caused by fixed charges on the surfaces of the membranes (Rand, 1981). These forces are sensitive to the ionic strength of the bathing medium and have decay distances between 10-100 Å (Parsegian, 1973). Van der Waals forces of attraction exist between all molecules and arise from induced electric dipoles (Voet & Voet, 1990). Hydration forces are insensitive to the ionic strength of the bathing medium (Marra & Israelachvili, 1985; Afzal

Figure 3. The diagrammatic representation of the assembly of phospholipids in the lamellar phase: (Adopted from Rand & Parsegian (1992)). The blank spaces represent the solvent in which the phospholipids are allowed to phase separate.

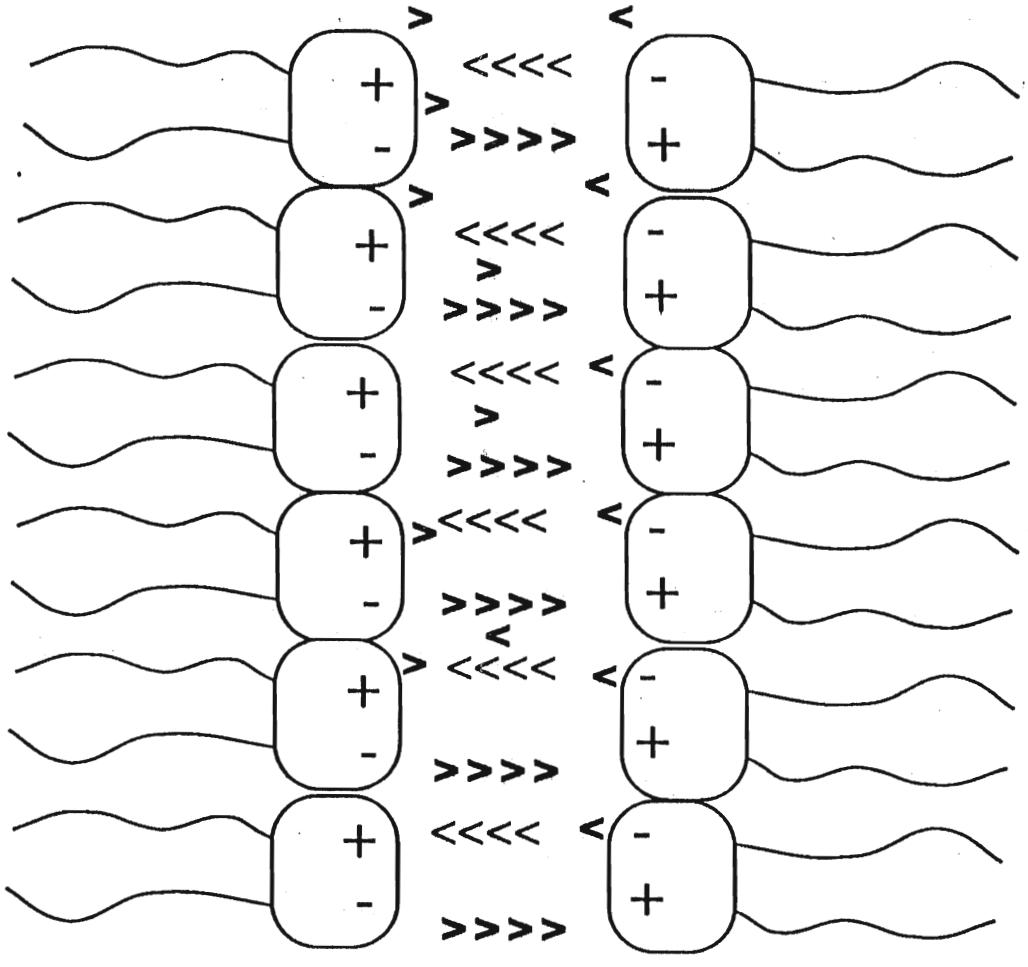

Assembly
Aggregation

et al., 1984; Lis et al., 1981), and have been shown to dominate close range interactions between surfaces.

The repulsive forces of hydration have been shown to dominate interactions at distances less than approximately 25 Å (Leiken et al., 1993; Parsegian et al., 1986; Rand, 1981; Rand & Parsegian, 1992). When electrically neutral phospholipids are placed in an aqueous environment, there is competition for water by the phospholipids hydrophilic head groups (LeNeveu et al., 1977). If water is removed from between the bilayers, then increased competition for available water will result in exponentially increasing repulsive forces as a function of decreasing bilayer separation (LeNeveu et al., 1977). The energetic cost of fully dehydrating lipid is quite large, and has been calculated for egg phosphatidylcholine to be approximately 6 Kcal/mol (Rand & Parsegian, 1989). It has recently been suggested that hydration forces have attractive components to them.

Hydration attraction forces are caused by the complementary orientation of water molecules between surfaces (Rand et al., 1988). The polar groups on the phospholipids will order the surrounding water molecules (Rand & Parsegian, 1992). These ordered waters will in turn influence the ordering of nearby water molecules causing the rearrangement of water molecules within the system (Rand et al., 1988). Due to the complimentary ordering of the water molecules that have correlations between them, hydration attraction forces will occur between the bilayers (Rand et al., 1988). A diagrammatic representation of hydration attraction is seen in figure 4 (Rand et al., 1988).

Figure 4. The diagrammatic representation of hydration attraction: the head groups on the phospholipids impart order on the surrounding water molecules. This ordering results in attractive forces of hydration between the phospholipid bilayers (Adopted from Parsegian et al., (1986)).



Where do the Large Forces Associated with Hydration Originate?

The attractive and repulsive forces of individual water molecules are less than their thermal energy suggesting that they will have little influence on any given system (Parsegian et al., 1986). However, the large hydration forces that have been measured result from the large numbers of water molecules that are involved (Parsegian et al., 1986). Even though each water molecule exerts little force within the system, the simultaneous removal of hundreds or thousands of water molecules, will amount to a significant force (Parsegian et al., 1986; Rand & Parsegian, 1989, 1992). The significance of water in living systems cannot be underestimated. Life can be broken down into a small amount of organic and inorganic materials that sit in approximately 80% water (Teeter, 1991). The requirement of hydration for enzyme function has also been well documented (Roe & Teeter, 1993).

Exclusion of Osmoticants from Proteins.

In protein chemistry, the techniques of salting-in and salting-out proteins from solution have long been used. Investigators have studied this phenomenon in order to determine the basic thermodynamic principles by which it occurs. The polymer PEG is considered to be one of the most useful agents for crystallizing proteins (McPherson, 1985). The underlying basis for this property is related to the exclusion of PEG from a volume around the protein.

The thermodynamic basis for salting-in and -out of proteins has been studied using many different solutes and proteins. Timasheff and coworkers have studied these phenomena with divalent cationic salts (Arakawa & Timasheff, 1984), amino acids (Arakawa & Timasheff, 1985), glycerol (Gekko, & Timasheff, 1981), and PEG (Arakawa &

Timasheff 1985; Bhat & Timasheff, 1992; Lee & Lee, 1987). The salting-in and out of proteins can be discussed with respect to the preferential hydration of the protein in the presence of solute molecules.

Preferential hydration is a term used to describe the hydration of a substance, such as a protein, in solution (Timasheff, 1992). Positive preferential hydration is defined as the state where there is more water than solute vicinal to the protein than is contained within the solvent solution (Timasheff, 1992). As a general rule, solutes that so "preferentially hydrate" proteins stabilize their structures (salt-out proteins)(Arakawa & Timasheff, 1985).

PEG is an excellent salting-out agent that shows little interaction with proteins (Bhat & Timasheff, 1992). Investigations have shown that several proteins are preferentially hydrated in the presence of PEG (Arakawa & Timasheff, 1985; Bhat & Timasheff, 1992). Evidence indicates that protein preferential hydration is negatively correlated with PEG concentration, and positively correlated with PEG molecular weight (Arakawa & Timasheff, 1985; Bhat & Timasheff, 1992). Bhat and Timasheff (1992) have recently concluded that polyethylene glycols are sterically excluded from the domains of many proteins. This exclusion is considered to be the impetus for the preferential hydration of proteins in the presence of polyethylene glycols (Bhat & Timasheff, 1992).

Studies of the preferential hydration of proteins allow the calculation of an effective volume of water surrounding proteins from which solutes are excluded (Schachman & Lauffer, 1949). This volume of exclusion (V_e) is defined as the volume of the water shell that surrounds the protein from which the centers of mass (radii of

exclusion, R_e) of the polymers are excluded (Schachman & Lauffer, 1949). Calculations show that the volume of exclusion of PEG from several proteins decreases as a function of increasing polymer concentration, and decreasing molecular weight (Bhat & Timasheff, 1992).

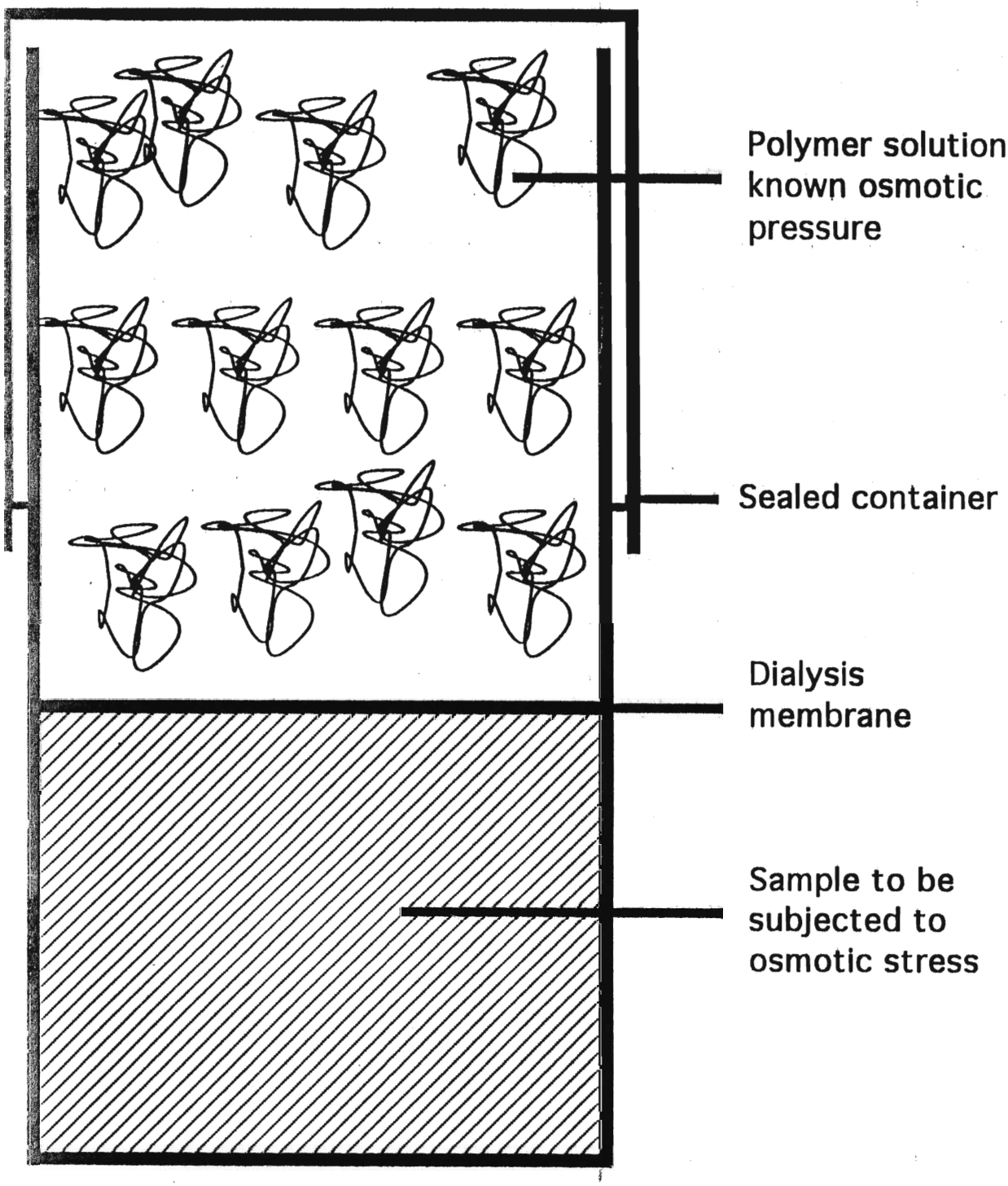
Osmotic Stress Application Associated with Osmotically Active Agents.

There is a long history of the investigation of the effects of inert solutes on proteins (e.g. Bull, 1981; Burk & Greenberg, 1930). The effects of hydration in several systems have been investigated by applying osmotic pressure controlled by solute solutions (e.g. LeNeveu et al., 1977; Rand et al., 1993). The generalized procedure for the application of osmotic stress with inert solutes is described below.

The osmotic stress technique requires a stressing solution, a sample solution, and a selectively permeable membrane (Fig. 5). The stressing solution is composed of water and some kind of osmotically active solute (osmoticant). An osmotically active solute is defined as any solute dissolved in the stressing solution that is restricted from the sample solution. The presence of the osmoticant will decrease the chemical potential of water within the stressing solution with respect to that of pure water (Bergethon & Simons, 1990). The osmotic pressure and the chemical potential of water of the stressing solution can then be measured independently by osmometry techniques.

The stressing and sample solutions are allowed to equilibrate through a membrane selectively permeable to water. Therefore, the chemical potential of water within the sample solution will be fixed by the chemical potential of water in the stressing solution after equilibration (Parsegian et al., 1986). The volume of the stressing

Figure 5. The diagrammatic representation of the application of the osmotic stress technique using osmoticants. Note: the semi-permeable dialysis membrane allows the unrestricted passage of water between the sample and stressing compartments, but restricts osmoticant (solute) passage.

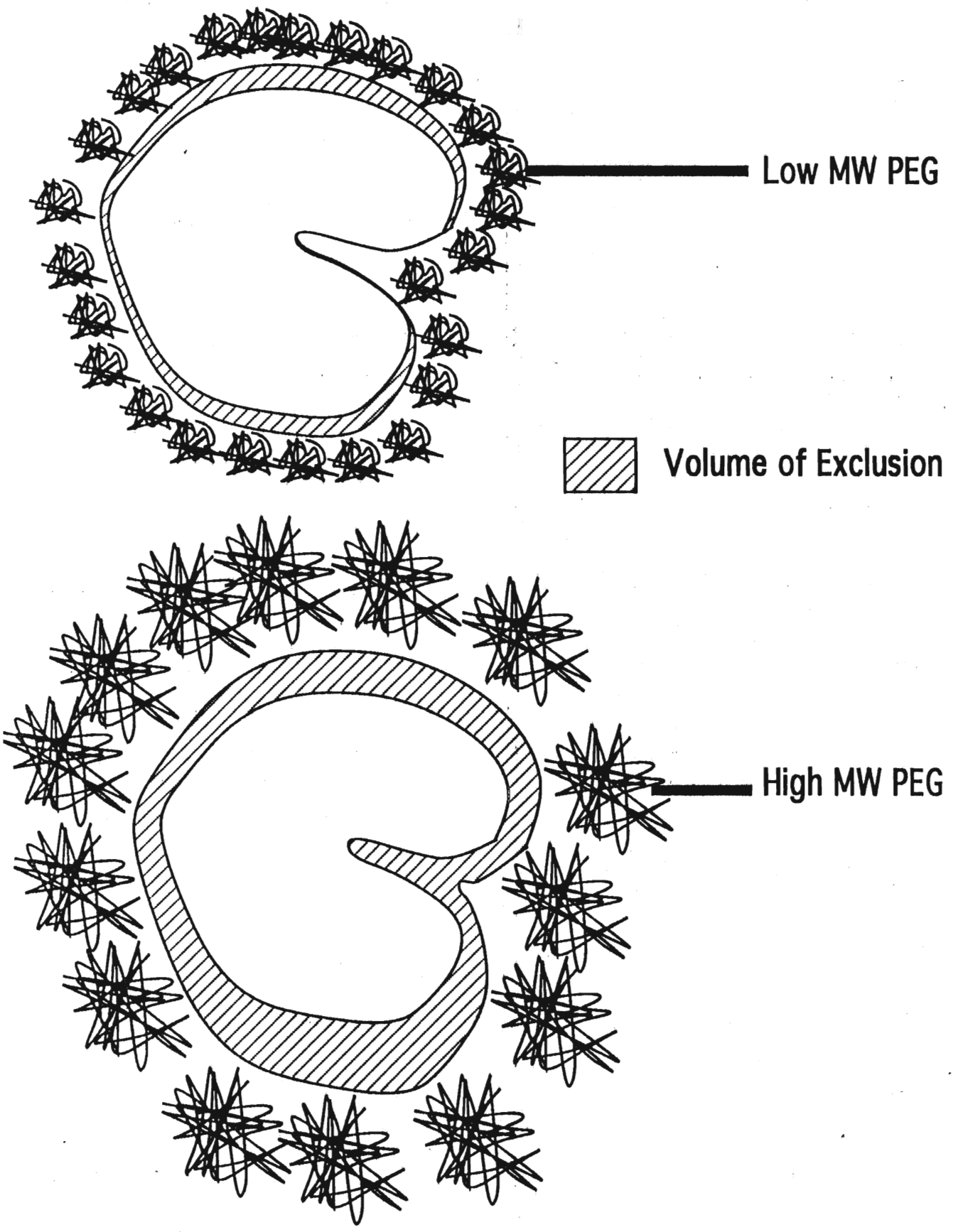

Polymer solution of known osmotic pressure
Sealed container
Dialysis membrane
Sample to be subjected to osmotic stress

solution is in great excess to that of the sample solution, so that equilibration will not affect its osmotic pressure (Parsegian et al., 1986). To ensure that the observed effects are solely due to osmotic pressure and not due to some characteristic of the osmoticant, it is important to use as many different solutes as possible (Parsegian et al., 1986).

The application of osmotic stress applies equally in systems that do not require physical membranes to separate stressing and sample solutions (Parsegian et al., 1986). In many systems, osmoticant exclusion from samples does not require physical membranes (Parsegian et al., 1986). Many polymers are excluded from phospholipid systems because they are too large to fit between bilayers (Rand & Parsegian, 1989). Similarly, several polymers are sterically excluded from the domains of many proteins (Bhat & Timasheff, 1992). Polymer exclusion surrounding many proteins is analogous to having a selectively permeable membrane surrounding the protein (Fig. 6). This is because the polymers are excluded from this volume, but water is not.

It is important to emphasize that solutes that are not excluded from the sample will not contribute to the osmotic pressure applied to the sample (Parsegian et al., 1986). The term chemical potential defines a property of matter that causes the flow of material from a region of high chemical potential to a region of lower potential (Bergethon & Simons, 1990). Implicit in this definition is a separation between regions of high and low chemical potential (Bergethon & Simons, 1990). With respect to osmotic stress, if there is no exclusion from the sample, the addition of solute will not cause a chemical potential difference between the water in the stressing and sample solutions because it will be equally distributed throughout. For

Figure 6. The diagrammatic representation of the exclusion of PEG molecules from a volume surrounding hexokinase. Note: the boundary depicting the volume of PEG exclusion is analogous to a semi-permeable membrane surrounding hexokinase that allows the exchange of water between this volume and the bulk solution, but restricts the access of PEG.


Low MW PEG
Volume of Exclusion
High MW PEG

example, if a salt is diffusable between the stressing and sample solutions through the selectively permeable membrane, its addition will not create a chemical water potential difference between the sample and stressing solutions (Bergethon & Simons, 1990). Even partial restriction of solute between stressing and sample solutions will cause a proportion of chemical potential difference between the stressing and sample solution with respect to what it would be if the solute were totally excluded (Bergethon & Simons, 1990).

Solution Non-ideality as it Relates to Osmotic Stress Experiments.

In an ideal solution, the addition of solute will increase its osmotic pressure by the following equation (Bergethon & Simons, 1990):

$$\Pi = RT \bullet C \qquad (3)$$

where Π is the osmotic pressure of the solution, R is the universal gas constant, T is the absolute temperature, and C is the concentration of the solute in osmolality.

In reality, the osmotic pressure of the solution increases disproportionately to increases in solute concentration due to intermolecular interactions (Rice, 1967). This disproportionate increase is termed solution non-ideality (Rice, 1967). Solution non-ideality makes it impossible to calculate the osmotic pressure of the stressing solutions simply from the knowledge of their concentrations. However, the osmotic pressures of polymer solutions can be directly measured with vapour pressure and secondary osmometry techniques with phospholipids.

Measuring the Role of Hydration in a Variety of Biological Systems.

Hydration Effects on the Interactions Between Surfaces.

Phospholipid Bilayers.

The osmotic stress technique has been extensively applied to many pure and mixed phospholipid systems (for review see Rand & Parsegian, 1989). It provides a way to measure the forces between bilayers as a function of separation. Initially, this work involved measuring the separation between egg lecithin bilayers as a function of osmotic pressure (LeNeveu, et al., 1976, 1977; Parsegian et al., 1979). The results have shown exponentially increasing repulsive forces of hydration as a function of decreasing bilayer separation (LeNeveu et al., 1976, 1977; Parsegian et al., 1979). For neutral phospholipids (Afzal et al., 1984; Lis et al., 1981; Marra & Israelachvili, 1985), and charged lipids below 20-30 Å separation (Cowley et al., 1978; McIntosh et al., 1990; Parsegian et al., 1991), hydration repulsive forces have been found to be insensitive to ionic strength. This observed insensitivity to the ionic strength of the medium clearly differentiates hydration forces from electrostatic forces.

Osmotic stress studies on phospholipid bilayers are important in characterizing the forces between membranes and also in establishing the osmotic stress technique as a valuable technique for hydration force investigations. The successful application of the osmotic stress technique to the study of hydration forces in phospholipid systems has led to its use with other systems.

DNA Double Helices.

DNA double helices display exponentially repulsive forces between 5 to 15 Å separation, with decay distances between 2.5 to 3.5 Å (Rau et al., 1984). These researchers have discovered that these forces are insensitive to the ionic strength of the bathing medium, which indicates that they are not electrostatic. Electrostatic double layer forces have

been shown to be influential in DNA packing only beyond 15 Å (Rau et al., 1984). These forces are remarkably similar to the hydration forces that occur between phospholipid bilayers, and suggests that hydration forces dominate interactions between DNA double helices at close separations (Rau et al., 1984).

Linear Polysaccharides.

Rau and Parsegian (1990) have recently been shown that exponentially repulsive forces exist between parallel arrays of helical polysaccharides which appears between 2 to 10 Å separation, and has a decay lengths of 3.3 Å. These researchers have discovered that the repulsive forces occur regardless of the charge on the polysaccharide, and are insensitive to the ionic strength of the bathing medium. These forces are very similar to the hydration forces between DNA double helices and phospholipid bilayers, and suggest that hydration forces dominate the interactions of polysaccharides at close separations.

Molecular Association.

Association of Proteins.

The association between proteins is well suited to the application of osmotic stress. In a system where the association of proteins is in equilibrium, the application of osmotic pressure would drive this equilibrium toward the more dehydrated form. As a general rule, proteins dehydrate as their contact surfaces come together (Kornblatt et al., 1993). Conversely, the application of hydrostatic pressure should shift the equilibrium to favor the more hydrated form (Snell et al., 1965). Kornblatt et al. (1993) have recently studied the association of cytochrome c-cytochrome b_5 and cytochrome c-cytochrome c oxidase in order to determine the relationship between their association and hydration.

Under appropriate conditions there will be an equilibrium between associated and dissociated cytochrome c and cytochrome b5 (Kornblatt et al., 1993). Osmotic stress shifts this equilibrium to favour the associated state (Kornblatt et al., 1993). These researchers have used osmotic stress measurements to determine that the associated enzymes dehydrate by two to four polymer-inaccessible water molecules. They also determined that the application of hydrostatic pressure to the proteins in solution (to increase hydration) promotes dissociation. This system provides a clear example of the importance of hydration to protein association.

Kornblatt et al. (1993) have also used the osmotic stress technique to study the association of cytochrome c-cytochrome c oxidase, and discovered some very unexpected behavior. They determined that the application of osmotic pressure shifts the equilibrium away from the association of these proteins. These researchers suggest that it is possible, but unlikely, that the glycerol might be binding to the protein. However, in the absence of glycerol binding, this research gives evidence for the very rare case where the associated state of cytochrome c-cytochrome c oxidase is hydrated by 12 more water molecules than the dissociated state.

Restriction Endonuclease.

Molecular biologists use high fidelity restriction endonucleases to cleave DNA at characteristic binding sites. The "Star activity" of a restriction endonuclease is a measure of cleavage fidelity and describes how often cleavage occurs at erroneous base pair sequences (Robinson & Sligar, 1993). Results obtained by Robinson and Sligar (1993, 1994) show that increasing osmotic pressure increases the star activity of *Eco* RI. This shows that water removal from the enzyme-DNA system

reduces *Eco* RI cleavage specificity. Alternatively, the application of hydrostatic pressure to a proteins in solution promotes hydration, and counteract the effects of osmotic dehydration (Snell et al., 1965).

Robinson and Sligar (1994) attempted to reverse the dehydrating affects of osmotic pressure by applying hydrostatic pressure simultaneously. Interestingly, it was discovered that the application of 400 bars hydrostatic pressure was necessary to eliminate the affects of 100 bars osmotic pressure. This finding highlights differences between the osmotic and hydrostatic pressure methods. The hydrostatic pressure method revolves around material compressibility to increase the density of water around a protein (Levitt, 1988; King & Weber, 1986 a,b; Pin, 1990). The osmotic stress technique is based upon moving water from one phase to another which does not necessarily involve compressibility (Parsegian, 1986). These findings clearly demonstrate the difference between these two methods and why correlations in results are at best fortuitous (Robinson & Sligar, 1994).

Hydration Affects on the Equilibria of Protein Conformations.

The Voltage Dependent Anion Channel.

Zimmerberg and Parsegian (1986) discovered that the Voltage Dependent Anion Channel (VDAC) is osmotically sensitive. The VDAC is a large protein (MW 31000-32000 Da) that is located in the mammalian mitochondrial membrane (Nakashima et al., 1986). Osmotic pressure causes a shift to the more dehydrated, closed form of the channel (Zimmerberg & Parsegian, 1986). The results show that the difference in polymer-inaccessible volume between the open and closed conformations of the channel to be between 22000 and 48000 $Å^3$ (Zimmerberg & Parsegian, 1986). This volume change is equivalent to

the dehydration of the closed conformation of the channel by 733 to 1600 polymer-inaccessible water molecules (Zimmerberg & Parsegian, 1986). Since the VDAC is sensitive to both transmembrane voltage and hydration, the results suggest that the VDAC might function in sensing, and/or controlling, the intracellular colloid osmotic pressure of mitochondria by coupling internal osmotic pressure with transmembrane voltage.

The Potassium Channel in the Squid Giant Axon.

Zimmerberg et al. (1990) have recently used osmotic stress techniques to investigate the conductance of potassium channels in the squid giant axon and discovered that it is osmotically sensitive. Their results show that osmotic pressure application causes decreased channel conductance. Osmotic stress calculations show that the channel is dehydrated by between 40 to 50 solute-inaccessible water molecules during its conformational change to its closed form (Zimmerberg et al., 1990). These findings show that apart from traditional voltage sensitivity, these channels are also osmotically sensitive. This has led these researchers to hypothesize that potassium conductance requires the hydration of some part, or parts, of the channel and that this hydration is independent of transmembrane voltage.

The Crayfish Giant Axons.

Rayner et al. (1992) have studied the sodium channel in the crayfish giant axon and discovered reduced conductance under osmotic pressure. Their osmotic stress calculations have shown that the more hydrated (open) channel is associated with 273 to 347 more solute-inaccessible water molecules than the dehydrated (closed) channel.

This study also shows that channel activation and inactivation are controlled by both voltage and solvent sensitive gates.

The Alamethicin Channel.

The alamethicin channel is a complex transmembrane pore that has been a rich model for voltage gated channels for many years (Hall et al., 1984). This channel undergoes transitions between several different conductance states, and has been investigated with the osmotic stress technique (Bezrukov & Vodyanoy, 1993). By probing this channel with different molecular weight polymers, it was possible to measure volume changes between conductance states, as well as gage the geometry of this channel (Bezrukov & Vodyanoy, 1993; Vodyanoy et al., 1993).

Osmotic stress measurements in the presence of polymers that are totally excluded from the lumen of this channel show that approximately 100 water molecules are taken up from the solution upon each transition to higher conductance states (Vodyanoy et al., 1993). Lower molecular weight polymers, that are not excluded from the channel, decreased channel conductance in a way that could be modeled as the result of increased micro viscosity of the channel's lumen (Bezrukov & Vodyanoy, 1993).

The geometry of this channel was investigated by using partially penetrating polymers and measuring access resistance (Bezrukov & Vodyanoy, 1993). Access resistance measures electrolyte access to the channel's opening (Laüger, 1976). These measurements indicate that the geometry of alamethicin channel's several conductance states resembles a closely packed cluster of pores rather than one pore of increasing conductance (Bezrukov & Vodyanoy, 1993).

Cytochrome c Oxidase.

Cytochrome c oxidase was the first solution protein studied using osmotic stress was cytochrome c oxidase (Kornblatt & Bon Hoa, 1990). Their results show that this enzyme undergoes a hydration/dehydration cycle during its turnover. These researchers hypothesize that cytochrome c oxidase requires the hydration, and subsequent dehydration, of 10 solute-inaccessible water molecules during its catalytic cycle. This hydration/dehydration cycle is necessary for the internal electron transfer to occur and corresponds to a conformational change in the enzyme.

Hemoglobin.

Colombo et al. (1992) used the osmotic stress technique to determine the influence of hydration on the binding of oxygen to haemoglobin. Their osmotic stress measurements show that the oxygenated state is hydrated by 60 more solute-inaccessible water molecules than the deoxygenated state. Colombo et al. (1992) point out that the large change in hydration between the oxy and deoxy states demonstrates the importance of water as an allosteric ligand for haemoglobin (Colombo et al., 1992). The large numbers of waters involved in the conformational change shows that hydration is a thermodynamically significant force in the oxygenation of haemoglobin. This work is very robust because it is based upon similar results obtained using several different osmoticants. However, some do dispute the findings (Bellelli et al., 1993). However, this investigation clearly highlights the potentially large energetic contribution of water to conformational changes within enzymes.

Calf Intestinal Adenosine Deaminase.

The effect of hydration on the catalytic cycle of calf intestinal adenosine deaminase has been investigated by the osmotic stress

technique (Dzingeleski & Wolfenden, 1993). These investigators compared the decreases in this enzyme's hydrolytic activity with osmotic pressure, dielectric constant, and viscosity. Their results show that the observed decreases best correlate with osmotic pressure changes. The authors suggest that calf intestinal adenosine deaminase exists in either a dehydrated or hydrated form, of which only the hydrated form is active. Their osmotic stress measurements show that at least nine additional solute-inaccessible water molecules are associated with the active form of this enzyme.

B to Z Transition in DNA.

Osmotic stress measurements have recently uncovered an osmotic sensitivity in the B to Z transition of DNA (Choe et al., 1994). Osmoticants drive the equilibrium of conformations towards the more dehydrated Z-form (Choe et al., 1994). During this transition, osmotic stress calculations show that the DNA is dehydrated by approximately 2.5 solute-inaccessible water molecules per base pair (Choe et al., 1994).

Hexokinase.

Rand et al., 1993 have used the osmotic stress technique to investigate the effects of hydration on a mixture of hexokinase isozymes. Their osmotic stress measurements show that the glucose bound conformation of hexokinase is dehydrated by 65 $\pm$ 10 polymer-inaccessible water molecules. These investigators used several PEGs with molecular weights higher than 2000, and obtained similar results for glucose binding using both equilibrium and kinetic measurements. A more detailed description of how the osmotic stress method was used to investigate the role of hydration in the

conformational change of hexokinase is described in the following section.

The Study of Hexokinase with the Osmotic Stress Technique.

As previously stated, PEG is excluded from an aqueous volume surrounding many proteins (Arakawa & Timasheff, 1984, 1985 a,b; Bhat & Timasheff, 1992; Lee & Lee, 1987; Timasheff, 1992). One may envision that this polymer-inaccessible volume is encompassed by a theoretical membrane separating the polymer solution in one compartment and the protein in another (Fig. 6). The polymer concentration in the outside compartment will control the water activity in this compartment and will aid in the removal of water from the protein compartment (Rand et al., 1993). If the polymer pressure in the outside compartment is Π, and n is the number of water molecules of total volume ΔV_w that move to the polymer compartment on glucose binding, then an osmotic work equal to $\Pi\Delta V_w$ will be done (Rand et al., 1993). Its contribution to the total change in free energy of glucose binding to hexokinase may be seen in the following equation (Rand et al., 1993):

$$\Delta G = -RT\ln K_d + nRT\ln(W) = constant \qquad (4)$$

where K_d is the GEDC of hexokinase, as described in an earlier section. The contribution of the water activity to glucose binding is related to the applied osmotic pressure by the following equation (Rand et al., 1993):

$$nRT\ln(W) = \Pi\Delta V_w \qquad (5)$$

One can then measure the change in the GEDC as a function of osmotic pressure in order to obtain the change in polymer-inaccessible volume between the open and closed conformations of the enzyme (ΔN_w)

(Rand et al., 1993). This is represented in the following (Rand et al., 1993):

$$\Delta V_w = \frac{RT\Delta \ln K_d}{\Delta \Pi} = \frac{RT \ln(K_d^{\circ} \div K_d^{\Pi})}{\Delta \Pi} \quad \textbf{(6)}$$

where K_d^0 and K_d^{Π} are the GEDCs at zero and Π, respectively. $\Delta\Pi$ is the change in pressure between zero and Π. This change in the volume of water may be related to the change in the number of water molecules by dividing by the volume of one water molecule of normal density. This allows one to determine ΔN_w.

Aims of the Present Research.

Previously, it was shown that approximately 65 ± 10 polymer-inaccessible water molecules are removed as the result of glucose binding to hexokinase (Rand et al., 1993). The present investigation is an extension of this earlier work. The specific aims are as follows:

1) to develop a procedure to reduce the previously observed variability in GEDC data. This will allow: A) a better understanding as to how the affinity for glucose of hexokinase changes as a function of osmotic pressure; B) give a precise measurement of the volume of solute-inaccessible water that is liberated as the result of glucose binding to hexokinase.

2) to use lower molecular weight polymers and produce higher osmotic pressures without increasing the viscosity of the polymer solutions, as compared to the higher molecular weight polymers.

3) to use lower molecular weight polymers to attempt to probe further into the polymer-inaccessible volume around hexokinase.

Materials and Methods.

The Enzyme and Experimental Solutions.

The hexokinase (Sigma) was essentially fraction II of Kaji (Kaji et al, 1961). The enzyme was reconstituted into glycylglycine buffer, which contained the following constituents: glycylglycine (GlyGly) 20 mmolar, 200 mmolar KCl. The buffer solution was adjusted to pH = 9.0 by the addition of potassium hydroxide. Glucose, PEG, and hexokinase solutions were prepared with this buffer. The enzyme solution was filtered through a 0.45 micron filter and did not show any loss of protein as measured fluorometrically. The concentration of hexokinase used in the assay solution was approximately 38.4 μg/ml. Glucose, PEG, and hexokinase solutions were prepared in this buffer.

The glucose used within this investigation was of the purest grade available (BDH biochemical). Polyethylene glycols of molecular weights 20,000, 10,000, 6,000, 3,000, 2,000, 1,500, 1,000, 600, and 300 were obtained from Fluka. PEG MW 8,000 was obtained from Union Carbide. All assay solutions were filtered with a 0.45 micron filter (Millipore) before use. The filtering had no effect on the concentrations of the polymer solutions, as measured through their refractive indices or, on selected samples, by vapor pressure osmometry. Dissolved gases were removed from the buffer and PEG solutions with an aspirator and side arm flask for a period of five minutes, prior to experimentation.

The various molecular weight (M_r) PEGs have distribution sizes as follows: PEG MW 20000 (M_r >17000), PEG MW 10000 (11500> M_r <9500), PEG MW 8000 (9000 > M_r <7000), PEG MW 6000 (7000> M_r <5000), PEG MW 3000 (3300> M_r <2700), PEG MW 2000 (2200> M_r <1900), PEG MW 1500 (1600> M_r <1400), PEG MW 1000 (1050> M_r <950), PEG MW 600 (630> M_r <570), PEG MW 400 (420> M_r <380), and PEG MW 300 (315> M_r <285).

Hexokinase was added either to the buffer solution or to the polymer/buffer solution. This assay solution was allowed to thermally equilibrate for a period of 15 minutes in the fluorimeter prior the start of each experiment.

Fluorescent Spectroscopy Measurements.

Fluorescence measurements were taken with a Perkin Elmer LS 50 spectrofluorimeter with a holding cell whose temperature was held at 25 ($\pm$0.1)^{0}C with a circulating water bath. The excitation wavelength of 290 nm and emission wavelength of 340 nm were chosen in order to maximize the fluorescent signal from the sample. In order to eliminate photodegradation of the fluorophore, the excitation slit was set to 2.5 nm. The emission slit was set at 6.0 nm in order to maximize the signal-to-noise ratio recorded by the spectrofluorimeter. Under these conditions, no decrease in the fluorescent signal of hexokinase was observed due to photo-degradation over a 20 minute time period (data not shown).

Fluorescence experiments were performed by adding small volumes of glucose solutions to the cuvette in the spectrofluorimeter with a Hamilton micro-syringe. Due to the significant volume fraction of the solutions occupied by PEG, the concentration of glucose in the experimental solution was calculated in molality units (grams glucose/1000 g water). The sequence of glucose additions was as follows: 1 μl, 1 μl, 2 μl, 4 μl (50 mM glucose dissolved in buffer); 2 μl, 2 μl, 4 μl, 8 μl (100 mM glucose dissolved in buffer); and 2 μl, 4 μl, 10 μl (1000 mM glucose dissolved in buffer). The solution in the cuvette was constantly stirred with a micro-stir bar and additional mixing was performed with a polypropylene stirrer after each glucose addition.

Fluorescence measurements were recorded with respect to time on the fluorimeter. After each glucose addition the experimental solution containing hexokinase was allowed to mix and gain a consistent fluorescence signal for a period of at least 30 seconds. If the signal remained consistent over that time period, the subsequent glucose addition was made.

The fluorescence data were analyzed on a IBM-386 DX computer with the Fluorescence Data Manager program (Perkin Elmer). Plots of the relative fluorescence of hexokinase with respect to time were used to estimate the average fluorescence of hexokinase after each glucose addition. The section of the plot corresponding to each glucose addition was enlarged and the average fluorescence signal was visually estimated. This estimation was affected by the noise in the fluorescence recordings.

The noise in the fluorescent signal was always less than five percent of the signal, and increased with the presence of suspended particulate matter in the experimental solution. Suspended particulate matter was observed in the cuvette during all experiments, even thought steps were taken to eliminate these particulates (eg. filtering the solutions). By disregarding changes in the fluorescent signal that were obviously due to these suspended particulates, the fluorescent signal could be conservatively estimated with an error of two percent of the overall signal.

The error associated with the estimation of the average fluorescence reading is less than the variability observed between independent experiments under the same conditions (eg. osmotic pressure). Therefore, the error in the estimation of the average

intrinsic fluorescence due to the noise must only be one component of the overall variability in the equilibrium binding experiments.

These average fluorescence estimates were then converted to the change in the intrinsic fluorescence of hexokinase by subtracting the estimate of the initial fluorescence reading on the spectrofluorimeter, before the addition of any glucose. These values were then normalized by dividing by the maximum change in fluorescence during the experiment.

Many molecular weight PEG solutions are well suited to work in the ultraviolet range due to their minimal light absorption in this region (measured by a Beckman DU 50 spectrophotometer). However, PEG MW 1200, PEG MW 200, Methylated PEG MW 2000, fructose, arabinose, stachyose, and glycerol were all osmoticants excluded from investigation because they either competitively inhibited hexokinase, or showed too high absorbency at 290 nm. Only osmoticants whose absorbencies at 290 and 340 nm were under 10%, at 30 weight percent concentrations, were used within the present investigation. The presence of any molecular weight PEG used within this investigation did not change the maximum glucose induced fluorescence quenching of hexokinase.

Estimation of the Glucose Equilibrium Dissociation Constant of Hexokinase.

The estimation of the GEDC can be made with double reciprocal plots showing the inverse of the normalized change in fluorescence verses the inverse of the glucose concentration (Hoggett & Kellett, 1976; Zewe et al., 1964). These estimated values are obtained from the plots at the glucose concentration where linear fits to the data intersect with the x-axis (Hoggett & Kellett, 1976; Zewe et al., 1964).

However, in the present investigation, computer fitting methods are used to estimate the GEDCs due to their advantages over the estimations obtained with double reciprocal plots.

Estimating GEDCs with double reciprocal plots suffer from unequal weighing of all data points (Bliumenfeld & Tikhonov, 1994). Computer fitting to non-linear models weighs equally all data points (Bliumenfeld & Tikhonov, 1994). The linear transformation of the data that occurs in double reciprocal plots can also skew the error distribution in data collected (Bliumenfeld & Tikhonov, 1994). Because of these disadvantages, the data collected within the present investigation were fitted using a non-linear computer fitting technique.

Statistical Evaluation of the Relationships Between the log Glucose Affinity Ratios and Osmotic Pressure.

Testing the equality of two populations of regression coefficients, with values (x_1,y_1; x_2,y_2), involves the use of Student's *t* in a way that is analogous to testing for differences between two population means. The null hypothesis, H^0, is that there is no significant difference between the two population regression coefficients.

What is first required is the summation of the data in the form of:

$$\sum x^2 =$$
$$\sum xy =$$
$$\sum y^2 =$$

The slopes (b_1 and b_2) of both regression lines may be calculated from the following equation:

$$b = \frac{\sum xy}{\sum x^2}$$

The residual sum of squares for both lines can be calculated from the following equation:

$$\text{residual SS} = \sum y^2 - \frac{(\sum xy)^2}{\left(\sum y^2\right)}$$

The residual degrees of freedom are equal to n-2, and the pooled residual mean square is calculated by:

$$\left(s^2_{Y*X}\right)_p = \frac{(\text{residual SS})_1 + (\text{residual SS})_2}{(\text{residual DF})_1 + (\text{residual DF})_2},$$

The standard error of the difference between regression coefficients is:

$$s_{b_1 - b_2} = \sqrt{\frac{\left(s^2_{Y*X}\right)_p}{\left(\sum x^2\right)_1} + \frac{\left(s^2_{Y*X}\right)_p}{\left(\sum x^2\right)_2}},$$

From these values the test statistic may be calculated, and is written as follows:

$$t = \frac{b_1 - b_2}{s_{b_1 - b_2}}$$

The t-value calculated is significant if it is greater than or equal to the values found within the t-tables at the appropriate level of significance. This calculation is a statistical test to determine if the slopes of two regression equations are significantly different from a common population slope. A sample calculation can be seen in Appendix II.

Calculation of the Change in Polymer Inaccessible Water Upon the Binding of Glucose to Hexokinase.

As discussed in the literature review, the change in the numbers of polymer-inaccessible water molecules upon the binding of glucose to hexokinase (ΔN_w) is determined from equation 6. This equation relates changes in the GEDC at zero pressure to the GEDC at Π osmotic pressure (log glucose affinity ratio) with respect to the change in the osmotic pressure. By plotting the log glucose affinity ratios with respect to osmotic pressure, the slope of the line of best fit allows the calculation of the change in the volume of polymer-inaccessible water upon the binding of glucose to hexokinase.

Since the relationship between these variables is curvilinear in the presence of many PEGs, plots were produced between these variables, and lines were drawn by eye through the data points. Tangents to these curves were then estimated graphically in order to obtain their slopes at the measured osmotic pressures. The tangents to these curves allowed the calculation of ΔN_w. The change in the numbers of polymer-inaccessible water molecules was calculated by dividing the change in the volume of water by the volume of one water molecule.

Measuring the Osmotic Pressures of the Polymer Solutions with the Vapor Pressure Osmometer.

The vapor pressures of the polymer solutions were measured with a Vapor Pressure Osmometer (VPO)(model 5500XRS: Wescor). The polymer solutions were prepared by gravimetrically adding PEG to double distilled water (wt/wt). The calculation of osmotic pressures from VPO data is given in appendix I. The standard errors of the means for readings from the VPO on the same polymer solutions were always less than five milliosmoles/Kg.

Due to the characteristics of the thermocouple head of the VPO, measurements below 100 mOsmol/Kg show non-linearity in the relationship between solute concentration and vapour pressure (Wescor). Since the vapour pressure osmometer estimates solute concentration by the ideal (linear) relationship between dew point depression and solute concentration, then measurements below 100 mOsmol/Kg were estimated by linear extrapolation from measurements at higher concentrations, that are not in question.

Non-additivity of Solution Osmotic Pressure.

Preliminary results indicated that the osmotic pressures of glucose and PEG solutions are not additive. The non-additivity is PEG concentration dependent and indicates that glucose does not have access to all the water in the solution in the presence of PEG. Thus, the glucose concentration in the presence of PEG, is referred to as the effective glucose concentration, is higher than in its absence (standard glucose concentration). These measurements are given in milliosmolal units, as previously described. Calculation of the effective glucose concentration in the presence of PEG is essential to determining the glucose concentrations in the protein assay solutions.

The effective glucose concentration was determined by the following procedure: 1) a 200 mmolar glucose solution was prepared and its osmolality measured using the VPO; 2) each molecular weight PEG used within this investigation was dissolved in double distilled water, at each weight percent concentration studied (gm PEG/100 gm water), and its osmolality was measured with the VPO; and 3) each molecular weight PEG used within this investigation was dissolved in the 200 mmolar glucose solution, at each weight percent concentration studied (gm PEG/100 gm glucose solution), and its osmolality was measured

with the VPO. This gives the osmolalities of the glucose, PEG in distilled water, and the combined PEG/glucose solutions. With this information, the factor by which the standard concentration of glucose increases as a function of PEG concentration is calculated. This is seen in the following calculation.

This example uses actual results obtained within this investigation to demonstrate the concentrating effects of a 35 weight percent PEG MW 2000 solution on glucose. The dilute glucose concentration makes it is reasonable to assume that the PEG is concentrating the glucose, and not the reverse. The measured osmolality of the standard glucose solution is 205 mOsmol/Kg. The measured osmolality of a 35 weight percent PEG 2000 solution in distilled water is 1194 mOsmol/Kg. The 35 weight percent polymer solution prepared by dissolving PEG in the glucose solution is measured to be 1591 mOsmol/Kg. The effective glucose concentration within the glucose/PEG solution is equal to 1591 mOsmol/Kg - 1194 mOsmol/Kg = 397 mOsmol/Kg (Note: the addition of the polymer to the glucose solution will not increase the ideal osmolality of the glucose). However, the standard glucose concentration should be 205 mOsmol./Kg if the glucose has access to all the water within the system. Therefore, the proportional increase in the glucose concentration is, 397 $mOsmol*Kg^{-1}$/ 205 $mOsmol*Kg^{-1}$ = 1.94. This shows that 35 weight percent PEG MW 2000 concentrates glucose by a factor of 1.94 over its standard concentration.

This is the generalized method by which the factor increase in the standard glucose concentration is determined for each PEG at a particular weight percent concentration. Note that this procedure must be performed for each molecular weight PEG, at each concentration used

in the present investigation. Once these factor increases in standard glucose concentrations were determined for all the different weight percent PEG concentrations, linear fits were applied to the data. From the equation of the lines of best fit, the factor increases in the standard glucose concentrations were obtained and applied to the standard glucose concentrations. These calculations gave the values for the effective glucose concentrations in the polymer solutions that were used in determining the GEDC of hexokinase.

Calculation of the Amount of Glucose Inaccessible Water per PEG.

The non-additivity of osmotic pressure of glucose and PEG provides information that allows a calculation of how much water is made inaccessible to glucose as a function of PEG concentration. An example of how this is calculated for a 35 weight percent PEG MW 2000 solution, the same example as above, is as follows.

The experimental protocol for the following calculation requires the addition of 35 weight percent PEG into a dilute glucose solution (200 mmolar). In 1000 g of a 35 weight percent PEG solution, there is 350 g PEG and 650 g glucose solution. The nominal 200 mmolar glucose solution has an average VPO reading of 205.4 mOsmol./Kg. The weight (w) of glucose in a 205.4 mOsmol/Kg solution:

$$\frac{205.4 \text{ mOsmol glusose}}{1000 \text{ g } H_2O} = \frac{w \text{ mOsmol}}{65 \text{ g } H_2O} \qquad (7)$$

$$\therefore w = \frac{13.351 \bullet 10^{-3} \text{ Osmol } (180.16 \text{ g / Osmol glucose}}{65 \text{ g } H_2O} \qquad (8)$$

w = 2.41 g glucose. Therefore, within the 65 g of solution that the PEG is dissolved in, there exists 2.41 g glucose. Next, find the amount of water in the 65 g of solution:

$$65 \text{ g solution} - 2.41 \text{ g glucose} = 62.59 \text{ g } H_2O \quad (9)$$

Therefore, within the solution there is 62.59 g H_2O that the solutes have access to. If x = the water available to PEG, and y = the amount of water available to glucose, then:

$$x + y = 62.59 \text{ g } H_2O \quad (10)$$

Measurements indicate that in the absence of PEG the concentration of glucose is measured to be 205.4 mOsmol/Kg. However, in the presence of 35 wt% PEG the concentration of glucose is measured to be 397 mOsmol/Kg. The difference in these two measurements can be reconciled with respect to the amounts of water that the different solutes have access to.

In the absence of PEG, the glucose in solution has access to all of the water, or both x and y. However, in the presence of PEG, the glucose only has access to the amount of water designated y. Since the amount of glucose in both solutions is equal then, the following equation can be written:

$$205.4(x+y)=397(y) \quad (11)$$

but,

$$x+y=62.59 \text{ g } H_2O \quad (12)$$

solve for y and substitute into the previous equation:

$$205.4(x+62.59-x)=397(62.59-x) \quad (13)$$

Therefore, x = 30.21 g H_2O. Since we have 35 g PEG, then we must have 30.21 g H_2O/35 g PEG, or 0.86 g H_2O/PEG. This method may then be applied to the different concentrations of all the different molecular weight polymers. These values are contained in Appendix Tables I and

II, given in g/g and mole/mole units. This calculation provides a simple and effective method for describing the amount of water made inaccessible to glucose as a function of PEG concentration.

Measuring the Osmotic Pressures of the Polyethylene Glycol Solutions Using "Secondary" Osmometry.

Polyethylene glycol was obtained from Fluka and dissolved in 2 mmolar TES (Sigma) buffer (pH 7). Dry lipid samples (Avanti Polar Lipids) were placed in sealed vials with TES buffer, or PEG/TES solutions, and allowed to equilibrate for at least 36 hours in the dark (at approximately 23 °C). The lipid samples were mounted with excess buffer, or PEG/TES solutions, into X-ray sample holders and sealed between mica windows, 1 mm apart, with powdered teflon for X-ray spacing calibration. The samples were electrothermally held at 20 °C (± 0.5 °C). X-ray diffraction displayed only the lamellar phase, producing diffraction lines of the repeat spacing *d* (± 0.1 Å), of a one dimensional crystal (Rand et al., 1988). The X-ray cameras used within the present investigation were of the Guinier type, operating *in vacu*, using the $CuK\alpha_1$ line (λ=1.540 Å). The diffraction patterns were recorded photographically on direct exposure film (Kodak). These d spacings yield the osmotic pressures of these PEG/TES solution by the following procedure.

The osmotic pressures of solutions were measured by the technique of secondary osmometry using the multilamellar phases of stearyloleoylphosphatidylcholine (SOPC) (Rand & Parsegian, 1989)(Fig. 7). The first part of this procedure involves the direct measurement of the osmotic pressures of a series of solutions (LeNeveu et al., 1976). Once the pressures of the solutions are determined, one may then investigate the effect that osmotic pressure has upon the d spacings of

phospholipids. SOPC is placed in the solutions of known osmotic pressure, and the repeat d-spacings of the phospholipid bilayers measured with x-ray diffraction (Rand & Parsegian, 1989). This allows one to determine the d-spacings of SOPC as a function of known osmotic pressure. The second part of this technique involves placing SOPC in polymer solutions of known concentration, but unknown osmotic pressure, as described above. This will give the relationship between the d-spacing and polymer concentration. The two parts of the procedure give two pieces of information: 1) the relationship between d-spacing and osmotic pressure and; 2) the relationship between d-spacings and concentration of the solutions of unknown osmotic pressure. By relating equivalent d-spacings of the unknown osmotic pressure solutions to that of the known solutions, one can determine the osmotic pressures of the unknown solutions.

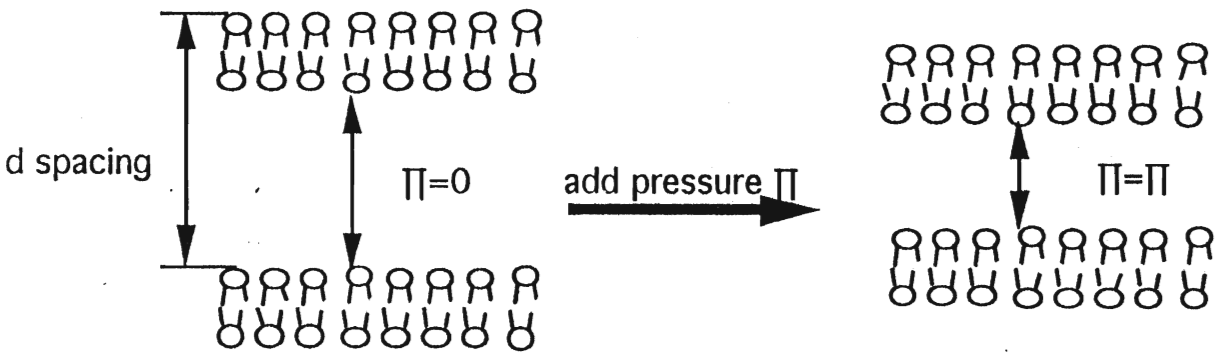
For example, if SOPC were placed in solution (K), of known osmotic pressure, it would adopt a multilamellar pattern with the repeat d-spacing, d. If SOPC were placed in solution (U) of known concentration, but unknown osmotic pressure, and it produced the same repeat d-spacing, then the osmotic pressure of both the solutions, (K) and (U) would be equal.

Molecular Modeling.

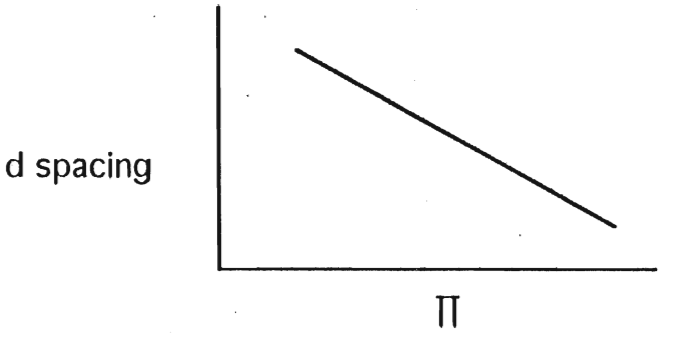
Molecular models were created using the computer program Quanta (Molecular Simulations, Waltham, MA). The crystal structures of both open and closed conformations of hexokinase 2 were obtained

Figure 7. The diagrammatic representation of the measurement of the osmotic pressure of a solution by "Secondary" osmometry with phospholipids.

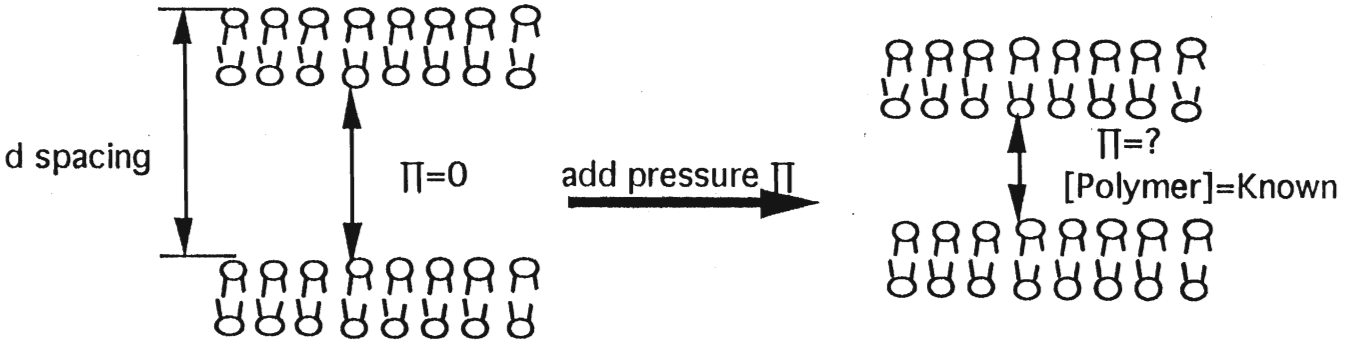
1) Know ∏: Find D-spacings of Phospholipids



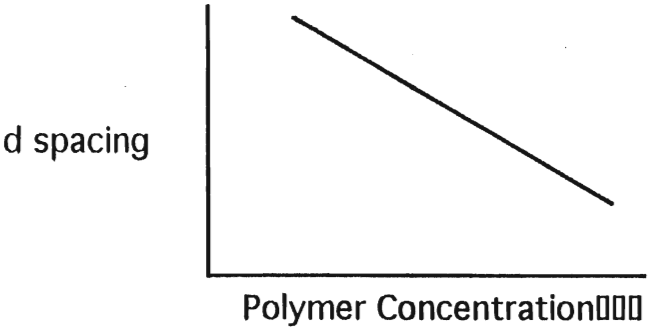
Obtain Relationship Between D-spacing and ∏



2) Unknown ∏, Known Polymer Concnetration-Find D-spacing



Obtain Relationship Between D-spacing and [Polymer]



Conclusion: at Equlilivant D-spacings, [Polymer] = ∏

from the Brookhaven Protein Data Bank, and glycines were substituted for any unknown amino acids. Solvent accessible surfaces were calculated for both open and closed conformations of the isozyme. The solvent accessible surface is defined as the area traced out by the center of a spherical probe (representing a solvent molecule), as it is rolled over the surface of the structure of interest (Connolly, 1983). The volume enclosed within the solvent accessible surface area is also calculated by the computer. Assuming no changes in protein density between the open and closed forms of the enzyme, differences in volume between the two conformations should be due to differences in the water structure surrounding the enzyme.

Initially, the probe radius of zero was used to determine the difference in the total enclosed volume between open and closed conformations of hexokinase. Subsequent calculations used three probe radii that were multiples of 2.8 Å (the diameter of one water molecule). Because the size of the probe changes, these calculations may not give an exact representation of the changes in the volume of water out to three hydration shells around hexokinase. These calculations may overestimate volume changes around the protein because the larger probes may be excluded from compartments around~~ hexokinase that probes the size of water would have access to. However, these calculations do give a general measure of the water volume changes between the open and closed conformations of hexokinase. The number of water molecules contained within the calculated volumes of water were determine by dividing by the volume of one water molecule of normal density (30 $Å^3$).

Results and Interpretations.

Reduction of Variability of K_d Measurements.

The first part of the present investigation involved the development of a procedure to reduce the scatter in the GEDC measurements. By systematically examining the various elements of the experimental protocol, a procedure was developed that reduced this variability. The methods added to accomplish this were: 1) degas the buffer and polymer solutions; 2) filter all solutions before use; and 3) use an excitation slit width of 2 nm, to eliminate photodegradation of the tryptophan fluorophore. Figure 8 shows an example of the variability in the double reciprocal plots before and after the new procedure was applied.

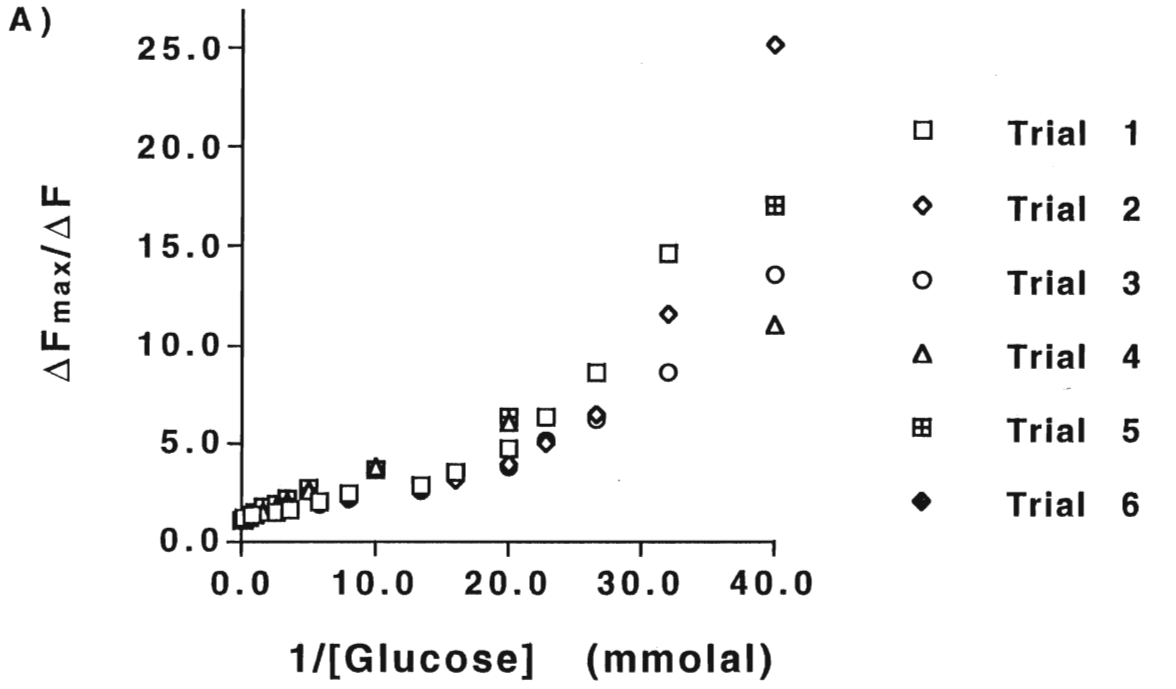
Osmotic Pressures Measured with the Vapor Pressure Osmometer.

Figure 9 shows the relationship between concentration and osmotic pressure measured by the VPO for several solutions containing different molecular weight PEGs. The concentrations of the PEG solutions measured by the vapour pressure osmometer are contained in Appendix Table III. These values, converted into osmotic pressures for solutions containing all the various molecular weight PEGs investigated are in Appendix Table IV (as calculated in Appendix I). These experimental measurements were used as measures of water activity for the hexokinase experiments.

Non-additivity of the Osmotic Pressures of Solutions Containing Glucose and PEG.

Figure 10 shows the ratio of the effective concentration of glucose in the presence of PEG MW 400 compared with the standard glucose concentration measured in the absence of polymer. Such ratios

Figure 8. Sections A) and B) show measured changes in the inverse normalized fluorescence of hexokinase with respect to the inverse of the concentration of glucose. Section A) shows measurements made before the adoption of the modified procedure to reduce the scatter in the measurements, and B) shows measurements subsequent to the adoption of the modified procedure. The experimental solution contains 38.4 μg hexokinase/ml buffer. The buffer was composed of 20 mM glyclyglycine with 200 mM potassium chloride (pH = 9.0). Fluorescence measurements were obtained with a Beckman LS 50 Spectrofluorimeter. Excitation and emission slit widths are 2.5 and 6 nm, respectively. Glucose was added to the cuvette with a Hamilton micro-syringe by the following addition protocol: 1 μl, 1 μl, 2 μl, 4 μl (50 mM glucose dissolved in buffer); 2 μl, 2 μl, 4 μl, 8 μl (100 mM glucose dissolved in buffer) and; 2 μl, 4 μl, 10 μl (1000 mM glucose dissolved in buffer). The experimental solution was constantly stirred with a mico-stir bar and further mixing was performed after each glucose addition with a polypropylene stirrer. Corrections were subsequently made for dilution dependent decreases in fluorescence.


A)
25.0
20.0
15.0
10.0
5.0
0.0
ΔFmax/ΔF
0.0
10.0
20.0
30.0
40.0
1/[Glucose] (mmolal)
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6

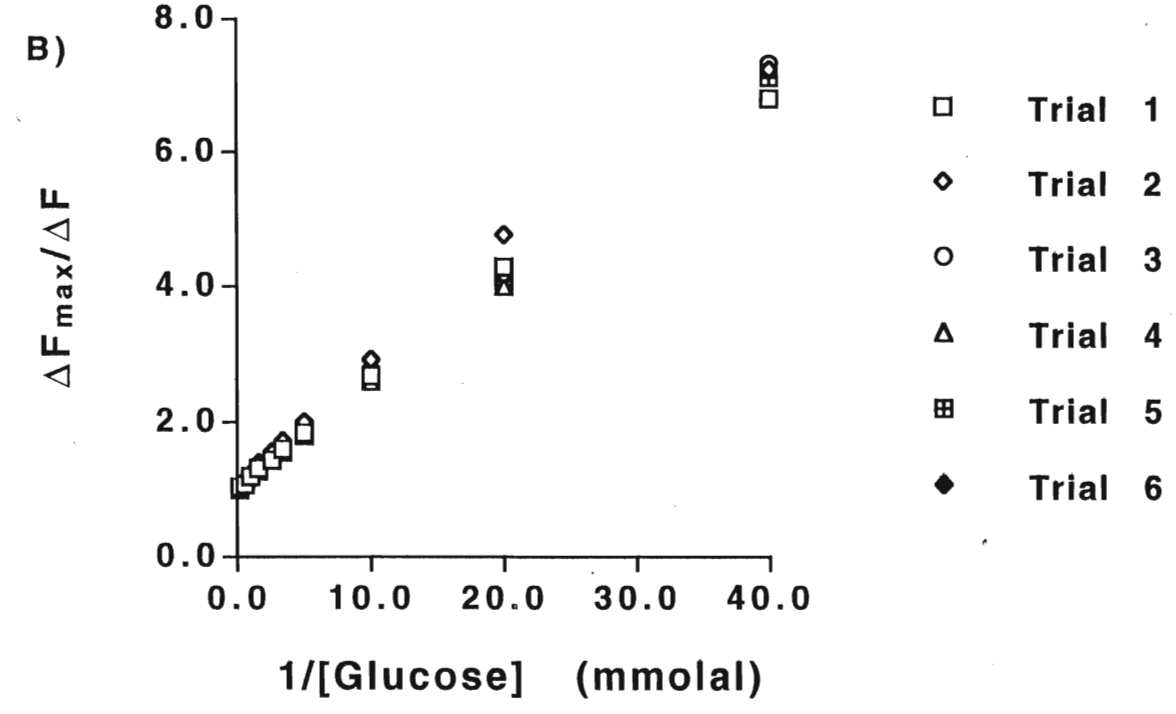

B)
8.0
6.0
4.0
2.0
0.0
ΔFmax/ΔF
0.0
10.0
20.0
30.0
40.0
1/[Glucose] (mmolal)
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6

Figure 9. Osmotic pressure measurements obtained with the vapour pressure osmometer (model 5500 XRS: Wescor) showing the relationship between PEG concentration (measured in molality) and the log osmotic pressure (given in dynes/cm^2). Measurements were made at 37 °C with PEG dissolved in double distilled water. Different symbols represent osmotic pressure measurements performed in the presence of different molecular weight PEGs. Each individual symbol represents the average of five independent measurements on the vapour pressure osmometer. The concentration of PEG was initially measured in weight percent (weight PEG/weight total solution) and later converted into molality. All values are contained in Appendix Table IV.

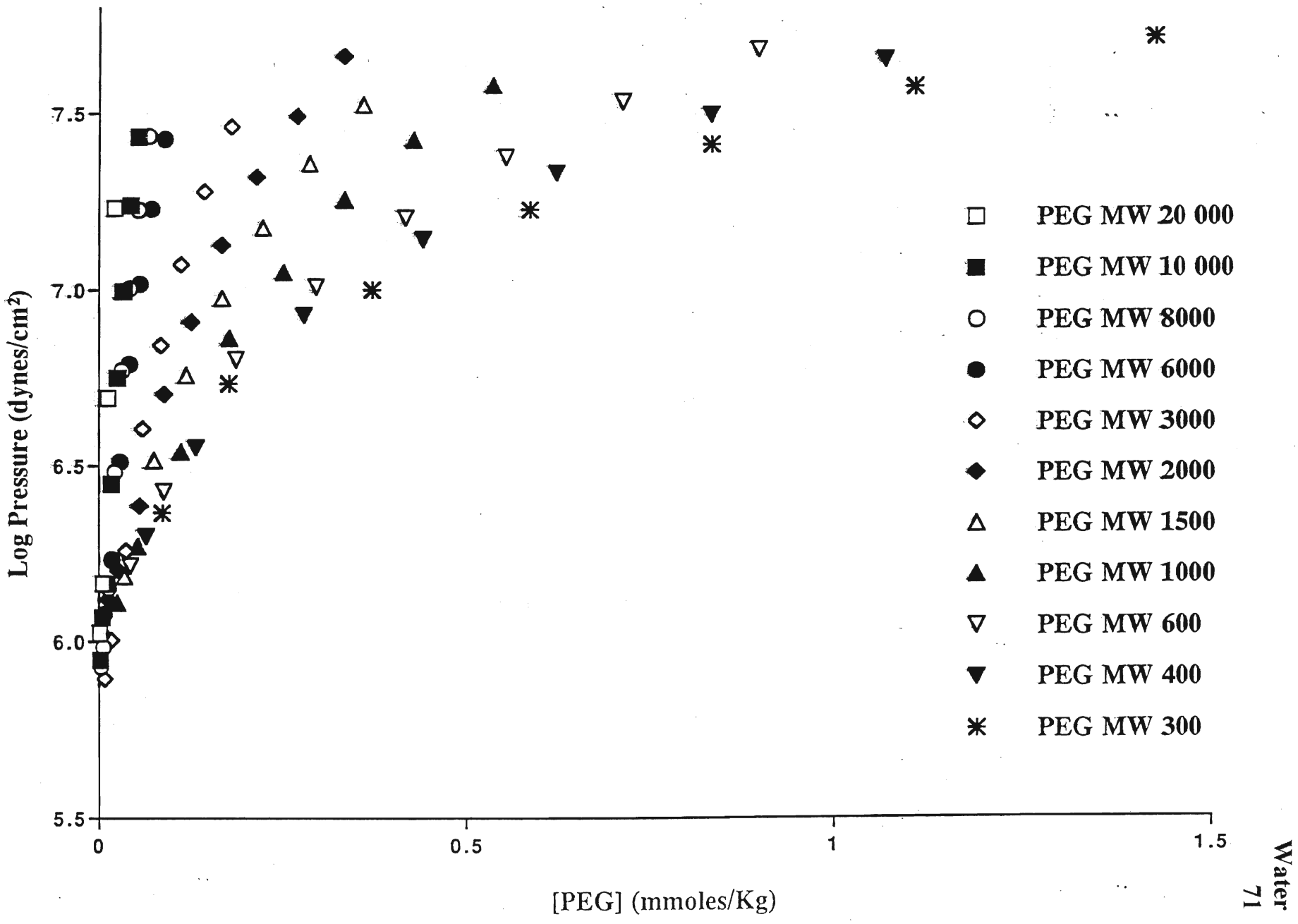
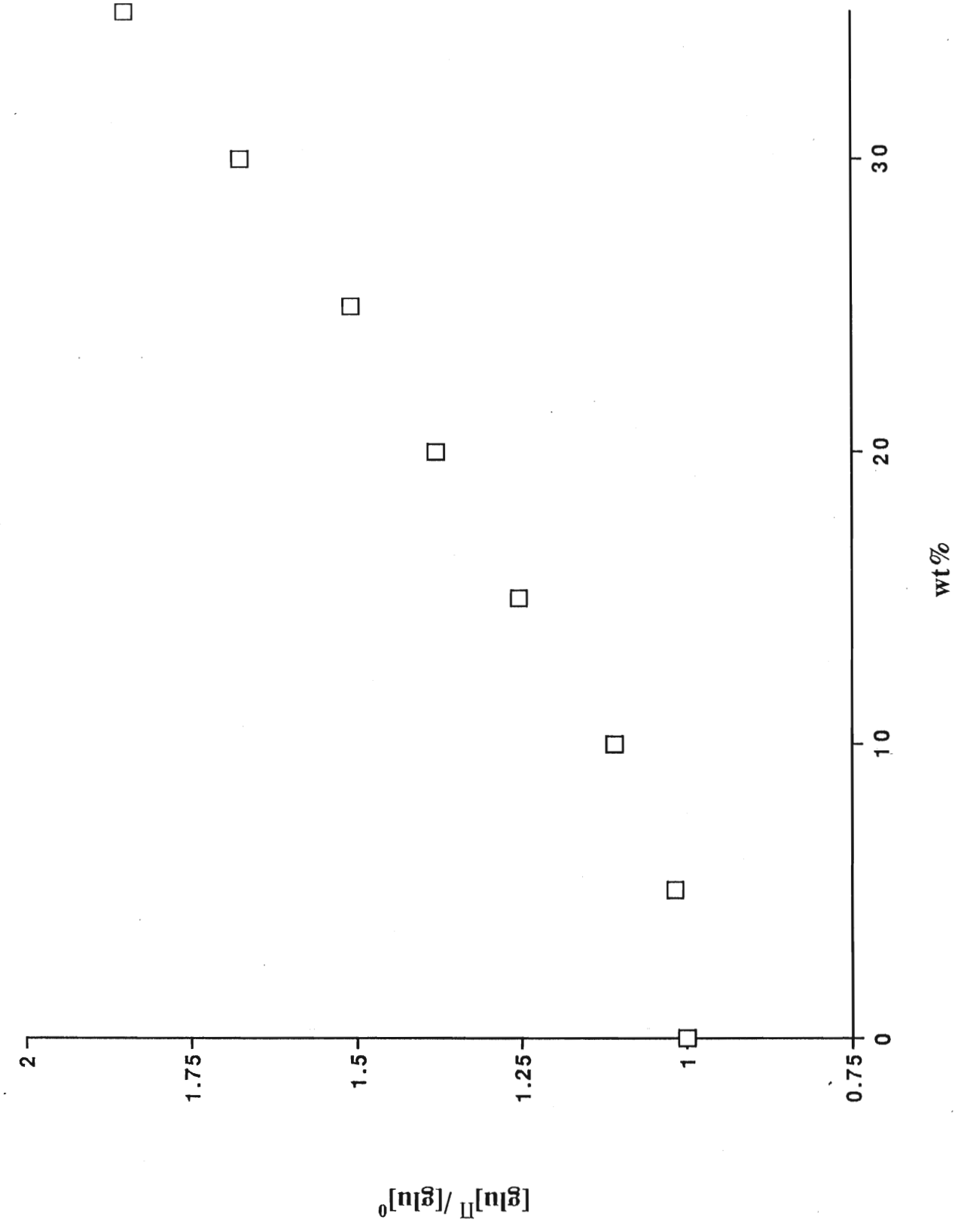

Log Pressure (dynes/cm²)
7.5
7.0
6.5
6.0
5.5
0
0.5
1
1.5
[PEG] (mmoles/Kg)
PEG MW 20 000
PEG MW 10 000
PEG MW 8000
PEG MW 6000
PEG MW 3000
PEG MW 2000
PEG MW 1500
PEG MW 1000
PEG MW 600
PEG MW 400
PEG MW 300

Figure 10. The ratio of the effective to the standard glucose concentration with respect to PEG MW 300 concentration (weight glucose/weight total solution). The effective glucose concentration as calculated by measuring the osmotic pressure non-additivity in the presence of PEG (as calculated in the materials and methods section). The standard glucose concentration is measured in double distilled water. Each data point is calculated from the averages of five independent measurements on the vapour pressure osmometer (model 5500 XRS: Wescor). Calculated ratios of the standard/effective glucose concentration in the presence of all the different MW PEGs investigated are contained in Appendix Table V.


[glu]Π /[glu]0
2
1.75
1.5
1.25
1
0.75
0
10
20
30
wt %

obtained for the other molecular weight polymers are found in Appendix Table V. All molecular weight PEGs investigated were found to concentrate the co-solute glucose. The results show that increases in PEG concentration cause increases in the effective glucose concentrations within the PEG/glucose solutions. For example, 35 weight percent PEG MW 400 nearly doubles the concentration of glucose in the co-solute solution. The results also show that the magnitudes of the increases in the effective glucose concentrations are similar, regardless of PEG molecular weight (Appendix Table V).

Amount of Glucose Inaccessible Water/PEG.

Increases in the effective glucose concentrations in the presence of polymer are due to decreases in the water accessible to glucose. Changes in the effective glucose concentrations allow the calculation of the ratio of how much glucose inaccessible water there is per amount of PEG. Appendix Tables I and II show these ratios in g/g and mole/mole units. Figure 11 shows a representative example, for PEG MW 400, of the relationship between the ratio of glucose inaccessible water/PEG (mole/mole) as a function of PEG concentration.

These results show two trends for all polymers investigated: 1) the ratio of glucose-inaccessible water/PEG decreases as a function of increasing polymer concentration, and 2) there is a nearly linear relationship between the ratio of glucose inaccessible water/PEG and polymer concentration. The linear regression coefficient values for the ratios of glucose inaccessible water/PEG as a function of increasing PEG concentration are as follows: $R^2 \geq 0.95$ for PEG MW 10000, 2000, 1500, 600, 300, and $R^2 \geq 0.80$ for PEG MW 8000, 6000, 3000, 1000 (figures not shown).

Figure 11. A representative example of the calculated weight of glucose-inaccessible water per weight of PEG MW 400. This and other calculated ratios for the different MW PEGs are contained in Appendix Table 1. Due to small standard errors in the data collected with the vapour pressure osmometer, these calculations were performed using the averages of five independent osmometry measurements, at each PEG concentration.

Inaccessible H2O/PEG (g/g)
1.8
1.6
1.4
1.2
1
0.8
5
10
15
20
25
30
35
Wt% PEG

Secondary Osmometry with Phospholipids.

Figure 12 displays osmotic pressure as a function of PEG concentration as measured by secondary osmometry with phospholipids (values are located in Appendix Table VI). The osmotic pressures of the polymer solutions increase as a function of increasing polymer concentration. Furthermore, the osmotic pressures of the polymer solutions systematically increase as a function of PEG molecular weight. For example, the osmotic pressures of solutions containing PEG MW 300 and 400 are lower than those containing PEG MW 1000 and 1500, at equivalent molality concentrations.

Comparison of Polymer Solution Osmotic Pressures as Measured by Vapor Pressure Osmometry and Secondary Osmometry with Phospholipids.

Figures 13 and 14 show comparisons of osmotic pressure as a function of PEG weight percent concentration measured by vapour pressure osmometry and secondary osmometry with phospholipids. The results prove that both methods are equivalent for the osmotic pressure measurements of solutions containing PEG MW 1500 and 1000. However, figures 15 and 16 show discrepancies in the osmotic pressures for solutions containing PEG MW 400 and 300 measured by phospholipid and VPO techniques.

The osmotic pressures measured by phospholipid and VPO techniques for solutions containing PEG MW 400 and 300 show two trends (Figs. 15 and 16). These are: 1) the phospholipid technique measures lower osmotic pressures than the vapour pressure technique for solutions below the concentration of 20 weight percent PEG, and 2) both osmometry techniques show equivalent osmotic pressure measurements for solutions of 30 weight percent PEG. These trends

Figure 12. The relationship between osmotic pressure and PEG concentration measured by "Secondary" osmometry with phospholipids. The multilamellar X-ray diffraction patterns of stearyloleoylphosphatidylcholine (SOPC) were measured in two mmolar TES (Sigma) buffer (pH 7) or PEG/TES solutions at 25 °C. The various symbols distinguish between osmotic pressures measured in the presence of different MW PEGs. The concentration of PEG was initially calculated in weight percent (weight PEG/weight total solution) and was later converted into molality. All measured values are contained in Appendix Table VI.

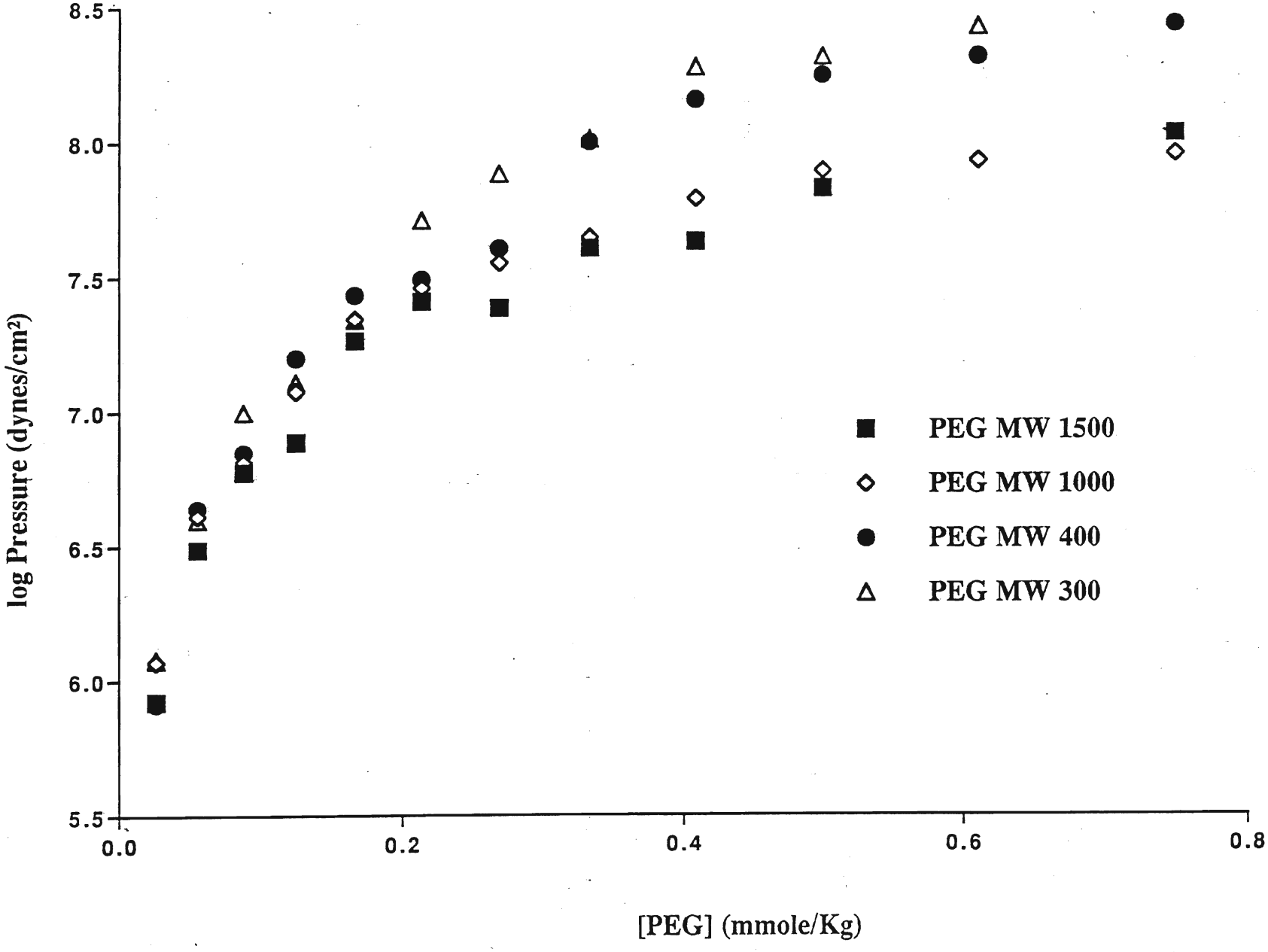
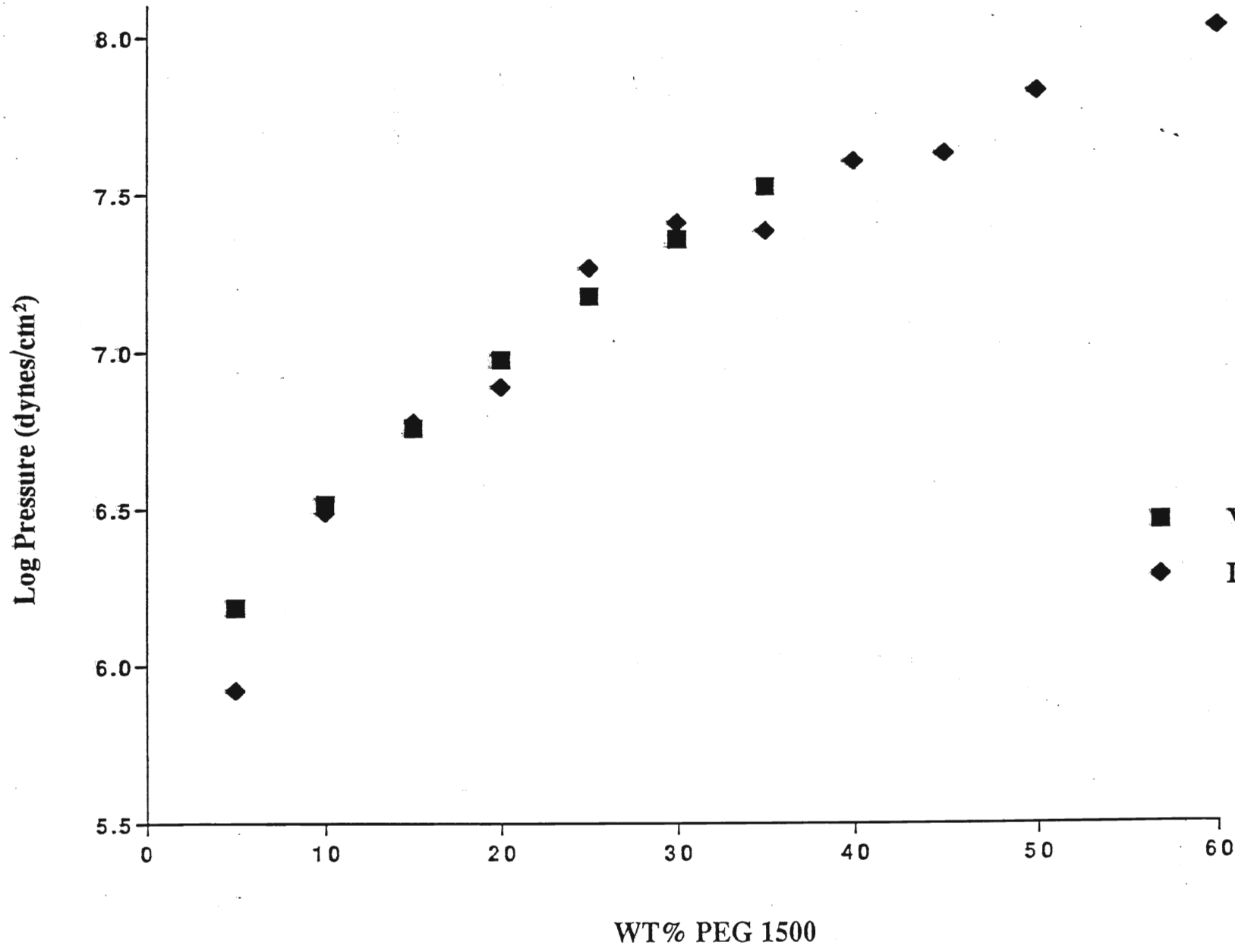
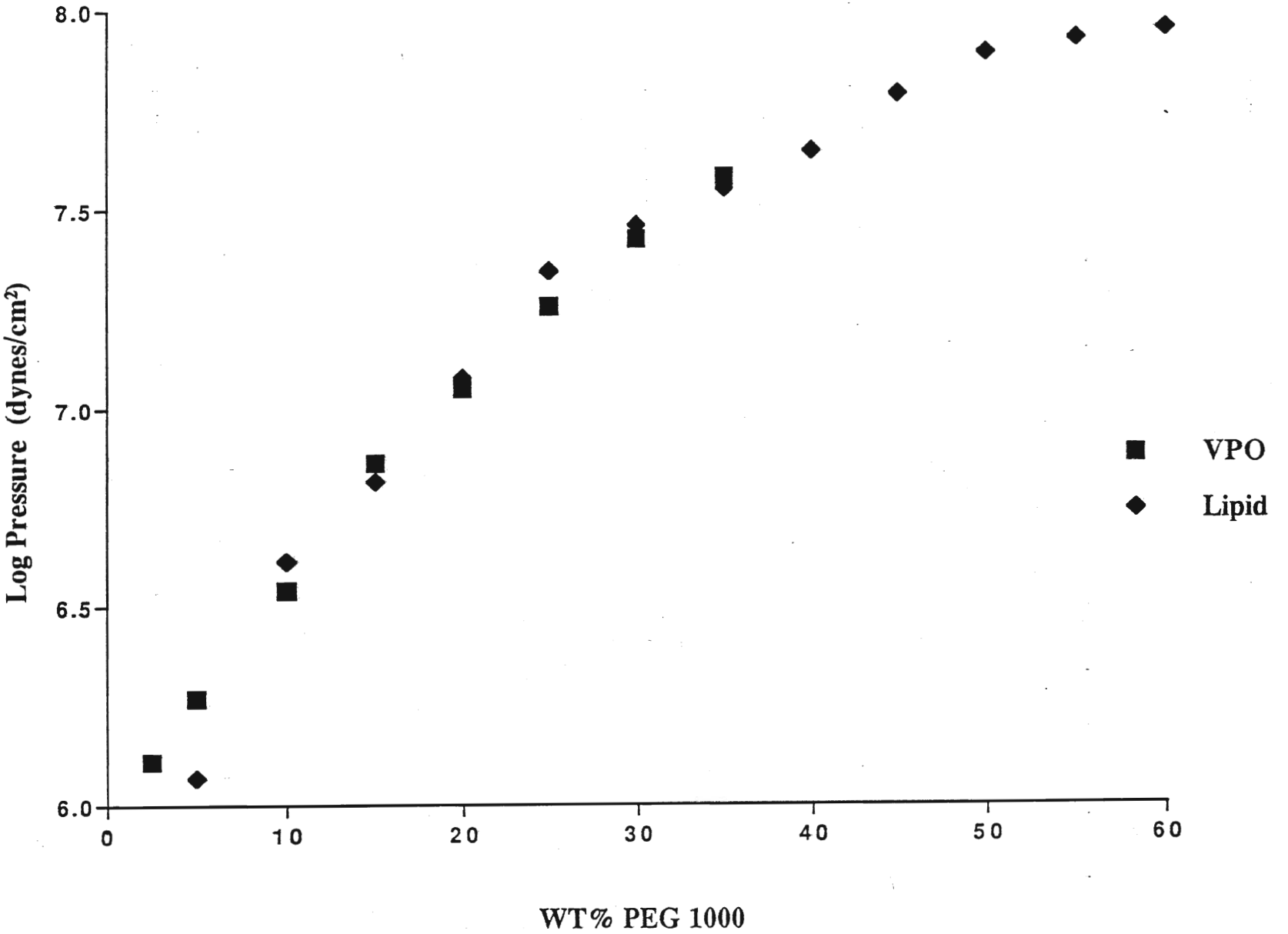

8.5
8.0
7.5
7.0
6.5
6.0
5.5
log Pressure (dynes/cm²)
0.0
0.2
0.4
0.6
0.8
[PEG] (mmole/Kg)
PEG MW 1500
PEG MW 1000
PEG MW 400
PEG MW 300

Figure 13. Comparison of the osmotic pressures measured with both the vapour pressure osmometer (model 5500 XRS: Wescor) and "Secondary" osmometry with phospholipids in the presence of PEG MW 1500. The osmotic pressure data presented obtained with the vapour pressure osmometer is calculated from the average of five independent measurements. The solutions examined were composed of PEG dissolved in double distilled water and the measurements wee performed at 37 °C. The osmotic pressure measurements using "Secondary" osmometry with phospholipids were performed using the multilamellar X-ray diffraction patterns of stearyloleoylphosphatidylcholine (SOPC) and were measured in two mmolar TES (Sigma) buffer (pH 7) or PEG/TES solutions at 25 °C. The concentration of PEG is given in weight percent (weight PEG/weight total solution).


8.0
7.5
7.0
6.5
6.0
5.5
Log Pressure (dynes/cm²)
0
10
20
30
40
50
60
WT% PEG 1500
VPO
Lipid

Water
81

Figure 14. Comparison of the osmotic pressures measured with both the vapour pressure osmometer (model 5500 XRS: Wescor) and "Secondary" osmometry with phospholipids in the presence of PEG MW 1000. The osmotic pressure data presented obtained with the vapour pressure osmometer is calculated from the average of five independent measurements. The solutions examined were composed of PEG dissolved in double distilled water and the measurements wee performed at 37 °C. The osmotic pressure measurements using "Secondary" osmometry with phospholipids were performed using the multilamellar X-ray diffraction patterns of stearyloleoylphosphatidylcholine (SOPC) and were measured in two mmolar TES (Sigma) buffer (pH 7) or PEG/TES solutions at 25 °C. The concentration of PEG is given in weight percent (weight PEG/weight total solution).


8.0
7.5
7.0
6.5
6.0
Log Pressure (dynes/cm²)
0
10
20
30
40
50
60
WT% PEG 1000
VPO
Lipid

Water
83

Figure 15. An example of the discrepancy between the osmotic pressures measured with the vapour pressure osmometer (model 5500 XRS: Wescor) and "Secondary" osmometry with phospholipids in the presence of PEG MW 400. The osmotic pressure data presented obtained with the vapour pressure osmometer is calculated from the average of five independent measurements. The solutions examined were composed of PEG dissolved in double distilled water and the measurements wee performed at 37 °C. The osmotic pressure measurements using "Secondary" osmometry with phospholipids were performed using the multilamellar X-ray diffraction patterns of stearyloleoylphosphatidylcholine (SOPC) and were measured in two mmolar TES (Sigma) buffer (pH 7) or PEG/TES solutions at 25 °C. The concentration of PEG is given in weight percent (weight PEG/weight total solution).

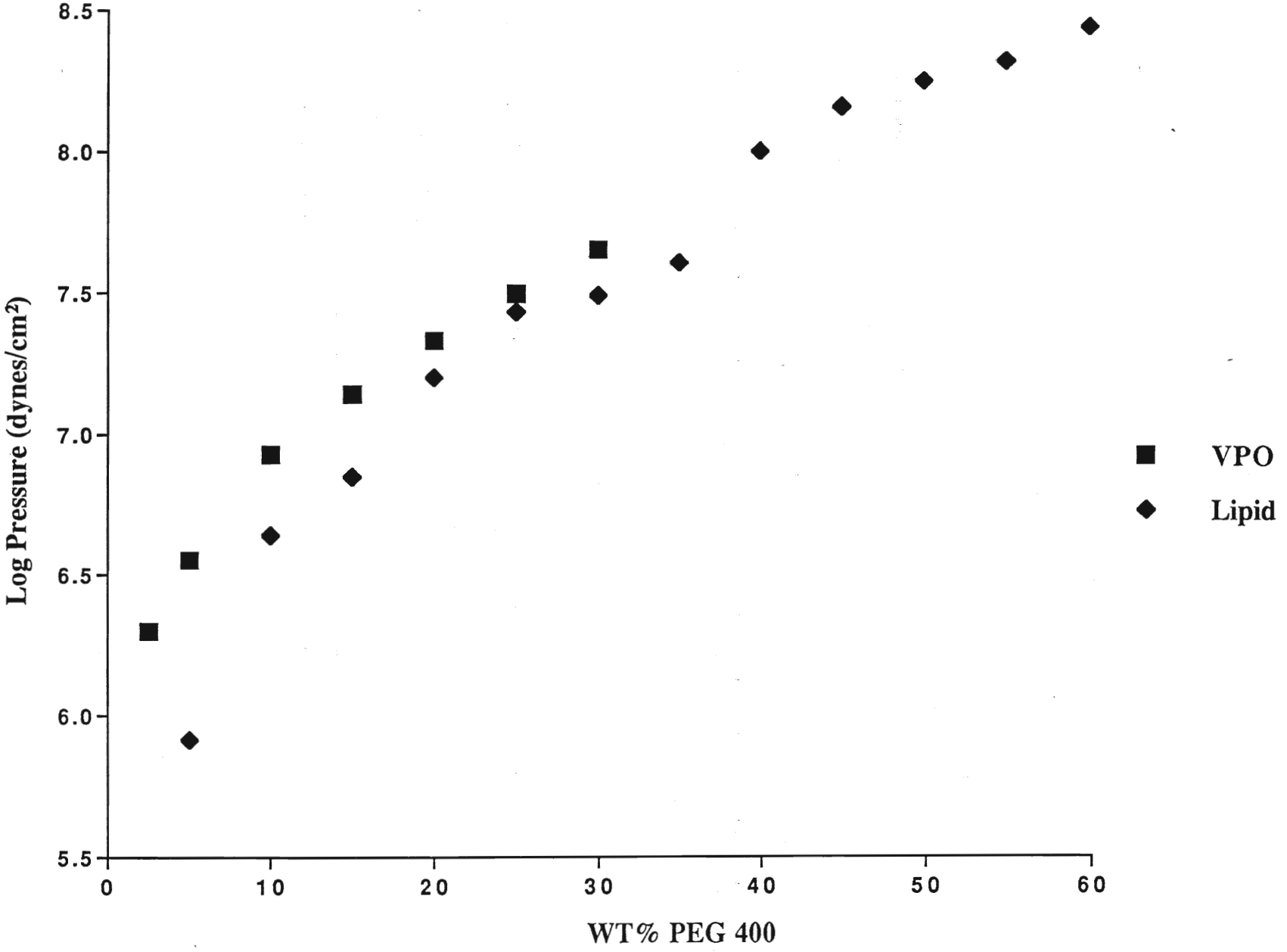
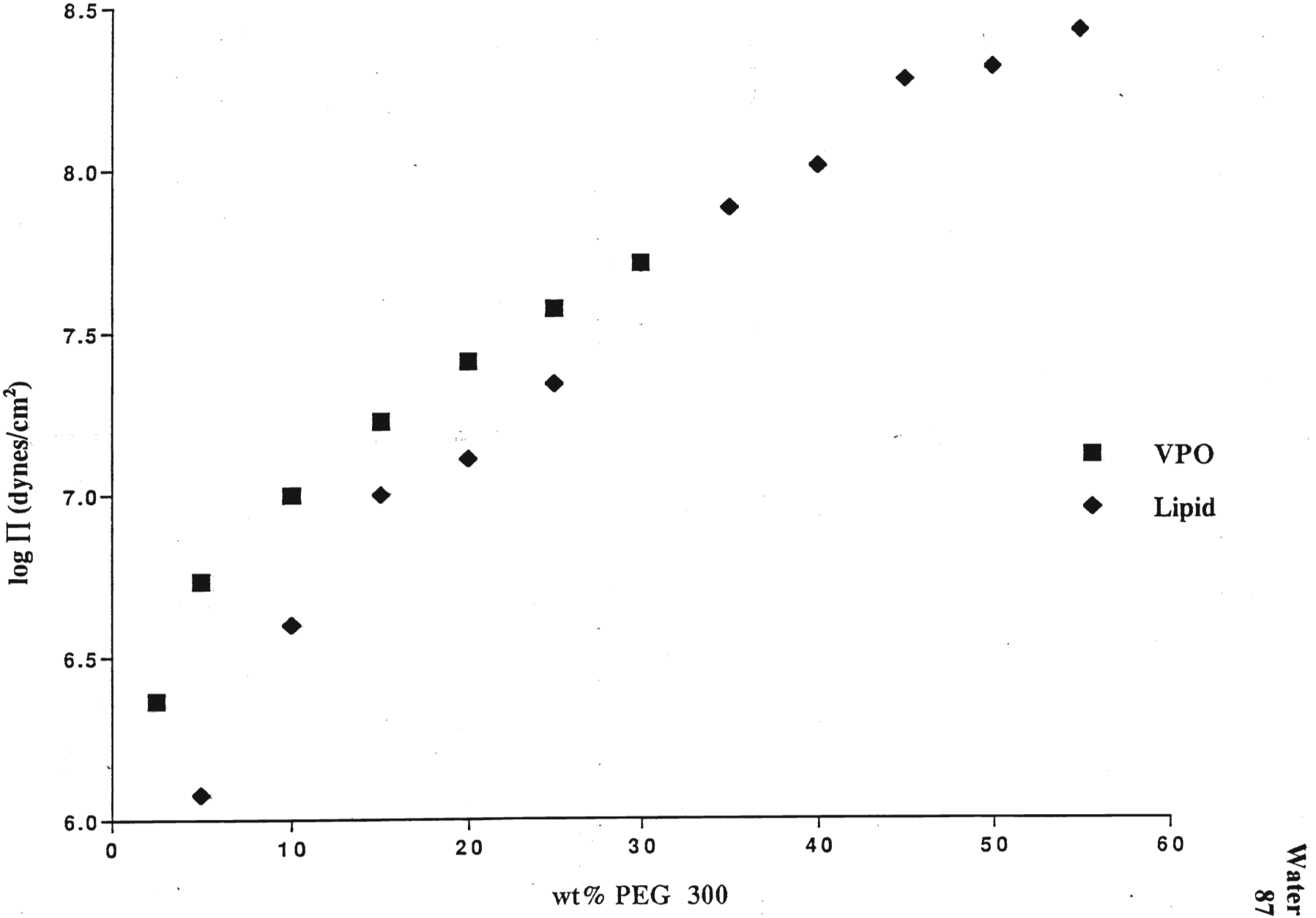

8.5
8.0
7.5
7.0
6.5
6.0
5.5
Log Pressure (dynes/cm²)
0
10
20
30
40
50
60
WT% PEG 400
VPO
Lipid

Figure 16. An example of the discrepancy between the osmotic pressures measured with the vapour pressure osmometer (model 5500 XRS: Wescor) and "Secondary" osmometry with phospholipids in the presence of PEG MW 300. The osmotic pressure data presented obtained with the vapour pressure osmometer is calculated from the average of five independent measurements. The solutions examined were composed of PEG dissolved in double distilled water and the measurements wee performed at 37 °C. The osmotic pressure measurements using "Secondary" osmometry with phospholipids were performed using the multilamellar X-ray diffraction patterns of stearyloleoylphosphatidylcholine (SOPC) and were measured in two mmolar TES (Sigma) buffer (pH 7) or PEG/TES solutions at 25 °C. The concentration of PEG is given in weight percent (weight PEG/weight total solution).


8.5
8.0
7.5
7.0
6.5
6.0
log Π (dynes/cm²)
0
10
20
30
40
50
60
wt% PEG 300
VPO
Lipid

Water
87

will later be discussed with respect to differences between the two osmometry techniques.

Measurement of the Glucose Equilibrium Dissociation Constant of Hexokinase.

Figure 17 displays a typical example of the measurement of the intrinsic fluorescence of hexokinase in a 30 weight percent solution of PEG MW 6000. These results show decreases in the intrinsic fluorescence of hexokinase within two seconds after glucose additions are made to the cuvette. As previously described, hexokinase binding to glucose induces a conformational change in its structure that causes a decrease in its intrinsic fluorescence (Hoggett & Kellett, 1976; Zewe et al., 1964). By relating the normalized decreases in intrinsic fluorescence to the concentration of glucose (assuming that hexokinase and glucose bind in a 1:1 ratio), the proportion of glucose bound with hexokinase is determined (see methods section for a more detailed description).

Figure 18 shows examples of double reciprocal plots relating decreases in normalized fluorescence to glucose concentrations. These double reciprocal plots provide a method for the estimation of the GEDC (see the materials and methods section). The results displayed in figure 18 clearly show that increasing polymer concentrations which correspond to increasing osmotic pressures of the stressing solution cause decreases in the GEDC of hexokinase.

Results indicate that the GEDC of hexokinase at zero osmotic pressure is 0.193 ±.009 mmolal (95% confidence limits, n=31). The results also show that the GEDC decreases as a function of increasing osmotic pressure in the presence of all PEGs investigated (Appendix Table VII). As previously described, changes in the GEDC, resulting from

Figure 17. A representative example of a typical data trace showing the decreases in the intrinsic fluorescence of hexokinase upon the addition of glucose in the presence of 30 weight percent PEG MW 6000. Large spikes on the data trace indicate when the glucose additions occurred. Fluorescence is measured in Arbitrary Units (AU). The experimental solution contains 38.4 μg hexokinase/ml buffer, 20 mM glycylglycine, 200 mM potassium chloride, pH 9.0 (25 °C). Fluorescence measurements were obtained with a Beckman LS 50 Spectrofluorimeter. Excitation and emission slit widths are 2.5 and 6 nm, respectively. Glucose was added to the cuvette with a Hamilton micro-syringe by the following addition protocol: 1 μl, 1 μl, 2 μl, 4 μl (50 mM glucose dissolved in buffer); 2 μl, 2 μl, 4 μl, 8 μl (100 mM glucose dissolved in buffer) and; 2 μl, 4 μl, 10 μl (1000mM glucose dissolved in buffer). The experimental solution was constantly stirred with a mico-stir bar and further mixing was performed after each glucose addition with a polypropylene stirrer. Corrections were subsequently made for dilution dependent decreases in fluorescence.

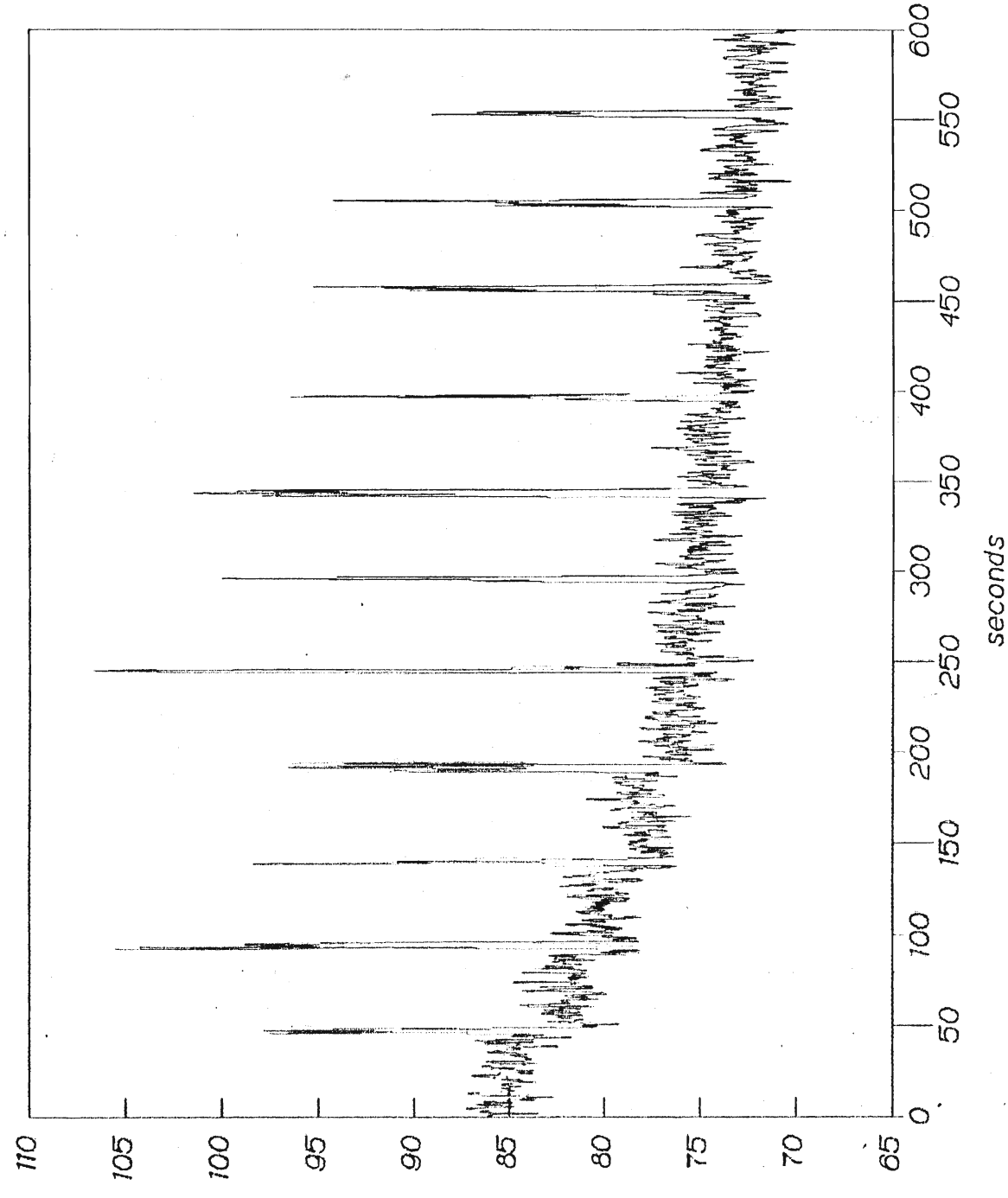
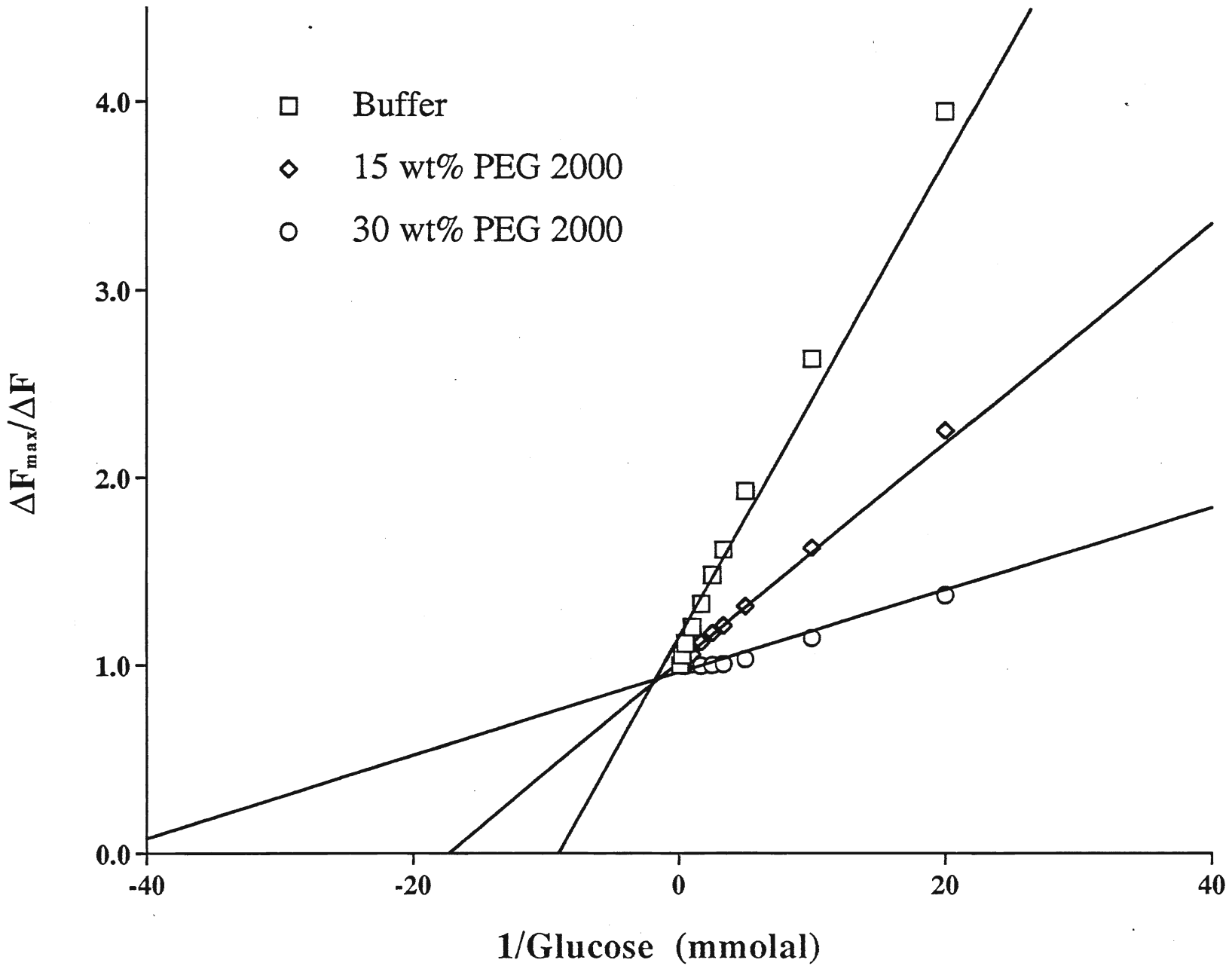

A.U.
110
105
100
95
90
85
80
75
70
65
0
50
100
150
200
250
300
350
400
450
500
550
600
seconds

Figure 18. The relationship between the reciprocal of the normalized decreases in the intrinsic fluorescence of hexokinase and the reciprocal of the glucose concentration. From these Lineweaver-Burk plots, it is possible to determine the glucose equilibrium dissociation constant of hexokinase at the concentration of glucose where the regression lines through the experimental data points intersect with the abscissa. Note: as the concentration of PEG MW 2000 increases, the glucose equilibrium dissociation constant decreases.


Buffer
15 wt% PEG 2000
30 wt% PEG 2000
4.0
3.0
2.0
1.0
0.0
-40
-20
0
20
40
ΔF_max/ΔF
1/Glucose (mmolal)

the application of osmotic pressure, reflect water's contribution to the glucose induced conformational change of hexokinase. ΔN_w is calculated by observing differences in the log glucose affinity ratios of hexokinase (log $K_d^{\circ}/K_d\Pi$) with respect to the applied osmotic pressure (equation 6).

The Effect of pH and Salt on the Glucose Equilibrium Dissociation Constant.

Experiments show that high concentrations of PEG lower solution pH (lowest observed pH = 8.43). The effects of decreasing pH were studied in order to verify that the observed decreases in the GEDC in the presence of PEG were not due to decreases in pH. Figure 19 displays the GEDC values as a function of pH. The results show that the GEDC of hexokinase does not vary within the pH range of 9 and 8.2.

As previously described, PEG concentrates co-solutes in solution. Thus, it is important to determine the effect on increased salt concentration upon the GEDC measurements of hexokinase (Fig. 20). The concentrating effects of PEG on potassium chloride are similar to glucose (data not shown), so it is reasonable to assume that PEG no more than doubles the salt concentration. Figure 20 shows doubling the concentration of potassium chloride has little effect on the GEDC values, compared to the effect of osmotic pressure. However, a small dependence of potassium chloride concentration on the GEDC measurements is noted.

The Relationship between the log Glucose Affinity Ratios and Osmotic Pressure.

Figure 21 shows the relationship between the log glucose affinity ratios of hexokinase as a function of osmotic pressure in the presence of several molecular weight PEGs (all calculated values are included in

Figure 19. The relationship between the pH of the buffer solution and the glucose equilibrium dissociation constant (GEDC) of hexokinase. The experimental solution contains 38.4 μg hexokinase/ml buffer, 20 mM glycylglycine, and 200 mM potassium chloride (25 °C). Changes in the pH of the buffer solution are as indicated in the figure legend. Fluorescence measurements were obtained with a Beckman LS 50 Spectrofluorimeter. Excitation and emission slit widths are 2.5 and 6 nm, respectively. Glucose was added to the cuvette with a Hamilton micro-syringe by the following addition protocol: 1 μl, 1 μl, 2 μl, 4 μl (50 mM glucose dissolved in buffer); 2 μl, 2 μl, 4 μl, 8 μl (100 mM glucose dissolved in buffer) and; 2 μl, 4 μl, 10 μl (1000mM glucose dissolved in buffer). The experimental solution was constantly stirred with a mico-stir bar and further mixing was performed after each glucose addition with a polypropylene stirrer. Corrections were subsequently made for dilution dependent decreases in fluorescence.

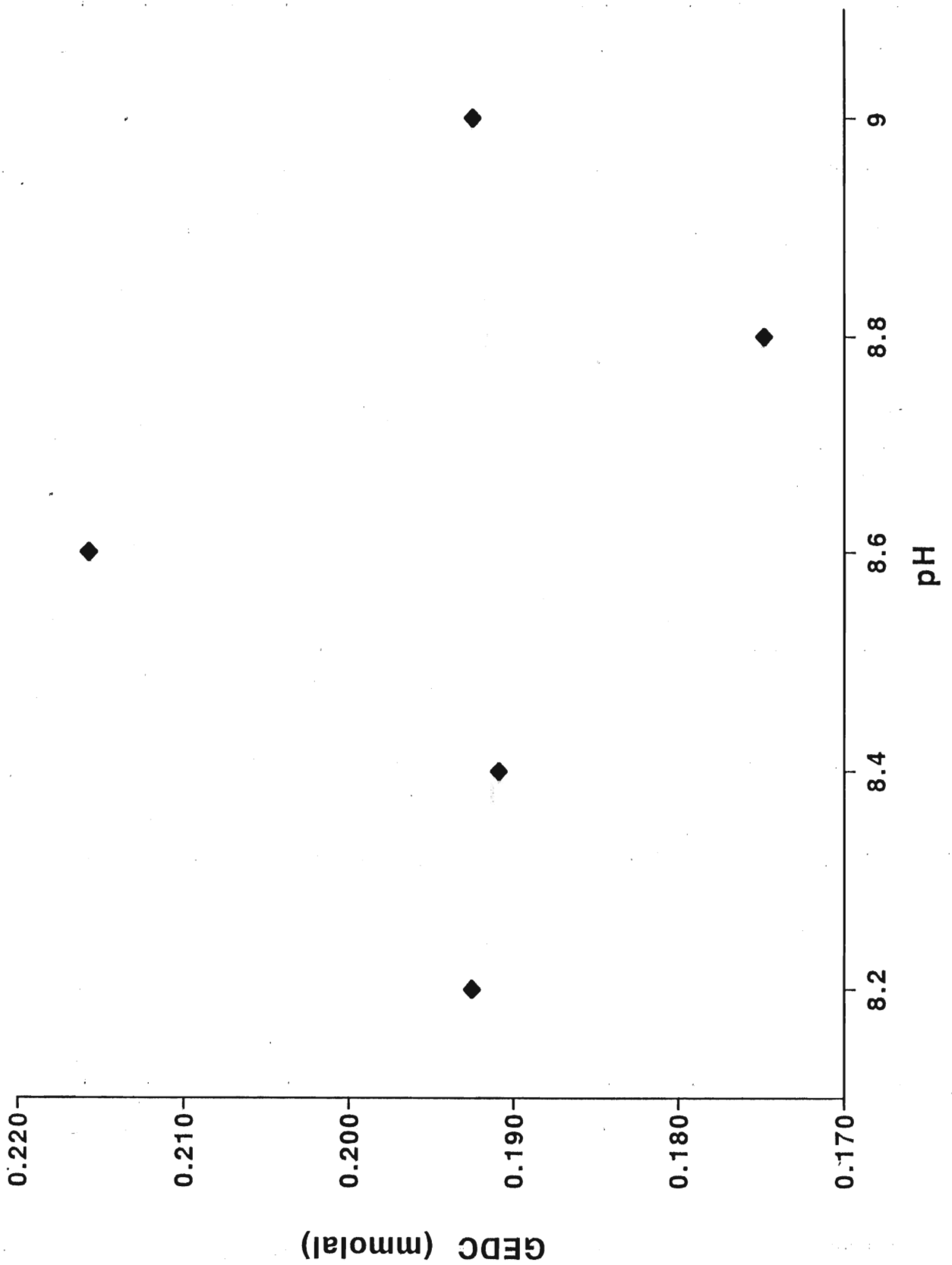

0.220
0.210
0.200
0.190
0.180
0.170
GEDC (mmolal)
8.2
8.4
8.6
8.8
9
pH

Figure 20. The relationship between the potassium chloride concentration in the buffer and the glucose equilibrium dissociation constant (K_d) of hexokinase. The experimental solution contains 38.4 µg hexokinase/ml buffer, 20 mM glycylglycine, at pH 9.0 (25 °C). Changes in the buffer's potassium chloride concentration of potassium chloride are indicated in the figure legend. Fluorescence measurements were obtained with a Beckman LS 50 Spectrofluorimeter. Excitation and emission slit widths are 2.5 and 6 nm, respectively. Glucose was added to the cuvette with a Hamilton micro-syringe by the following addition protocol: 1 µl, 1 µl, 2 µl, 4 µl (50 mM glucose dissolved in buffer); 2 µl, 2 µl, 4 µl, 8 µl (100 mM glucose dissolved in buffer) and; 2 µl, 4 µl, 10 µl (1000mM glucose dissolved in buffer). The experimental solution was constantly stirred with a mico-stir bar and further mixing was performed after each glucose addition with a polypropylene stirrer. Corrections were subsequently made for dilution dependent decreases in fluorescence.

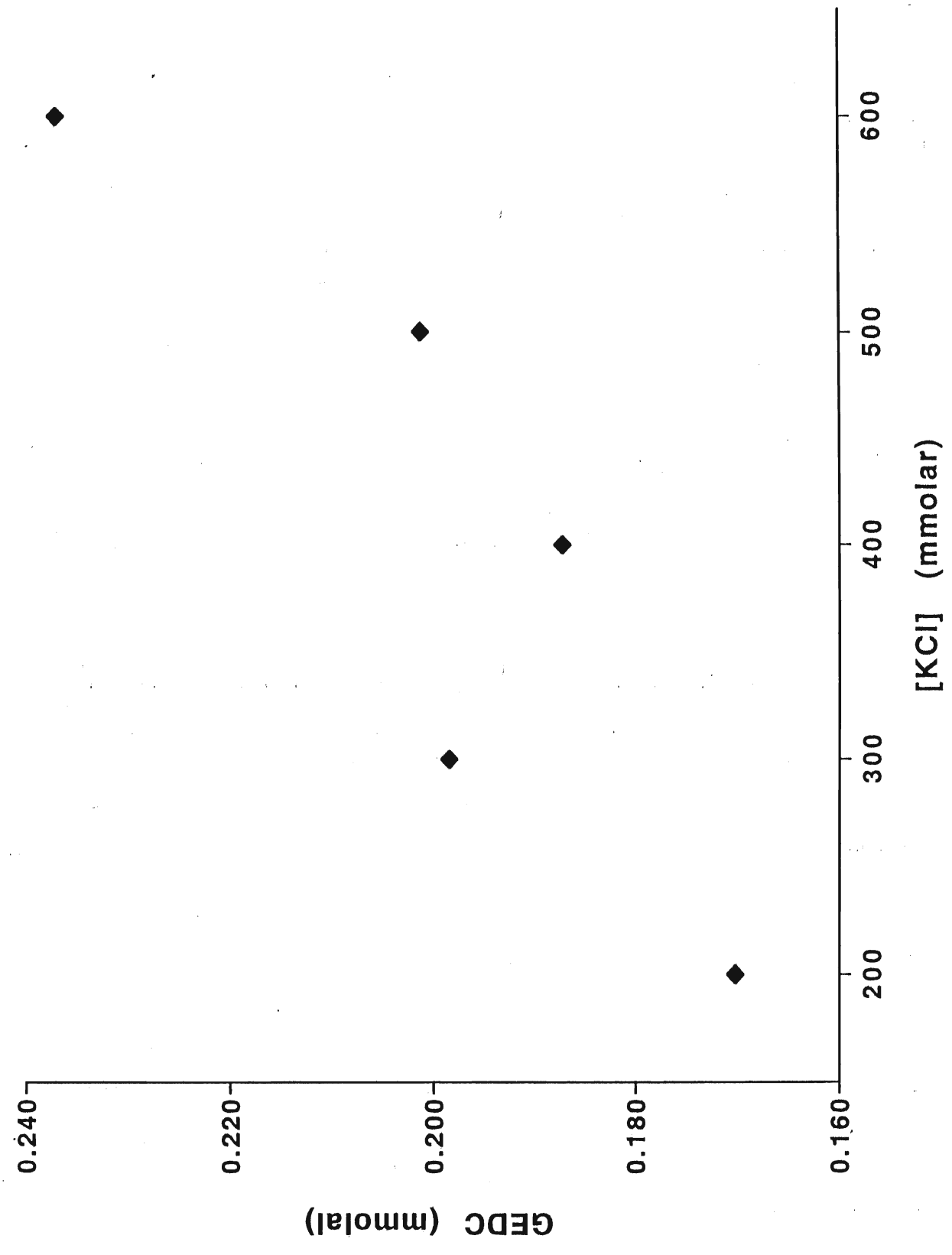
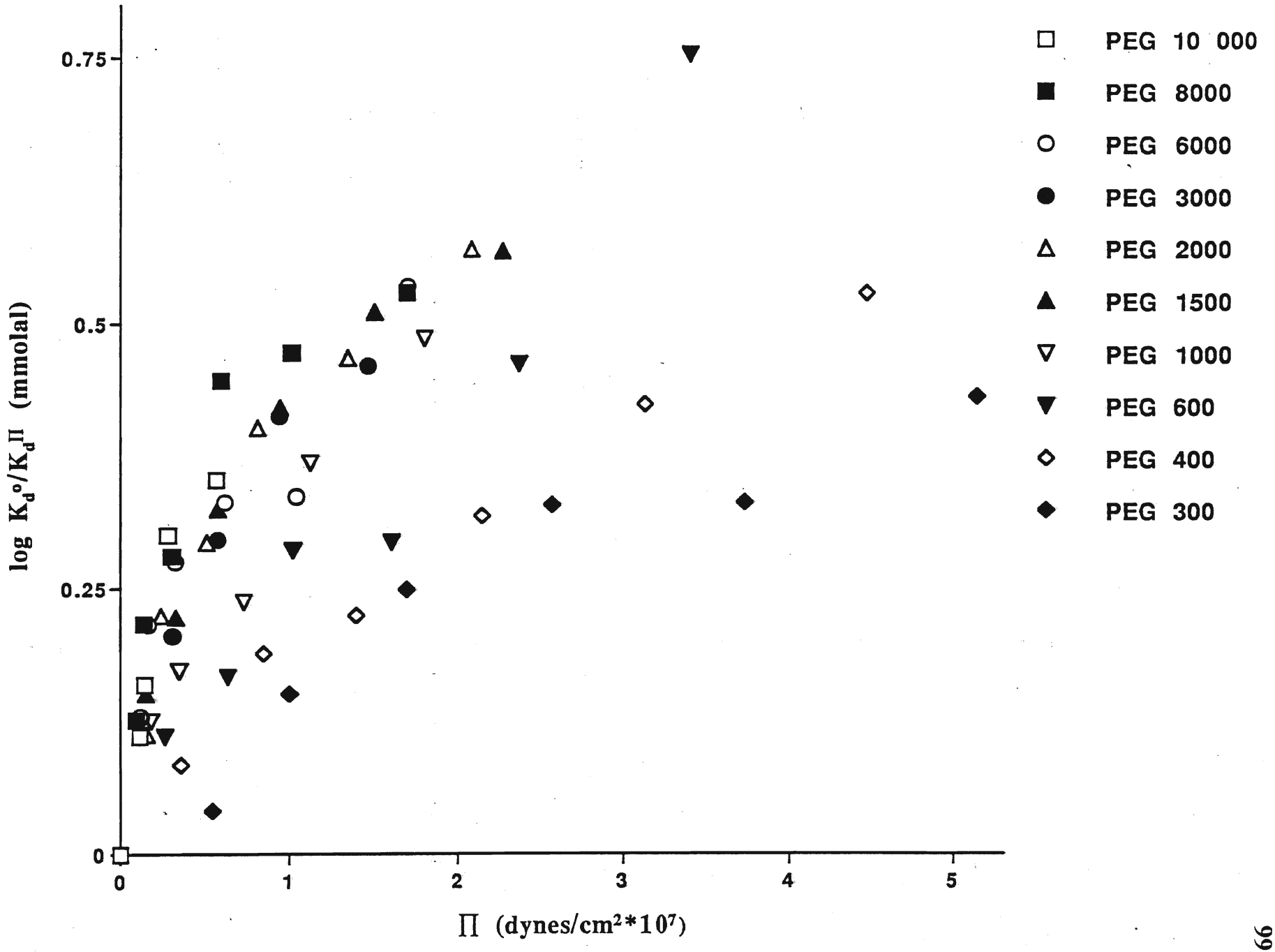

GEDC (mmolal)
0.240
0.220
0.200
0.180
0.160
200
300
400
500
600
[KCl] (mmolar)

Figure 21. The relationship between log K_d°/K_d^{Π} verses osmotic pressure. Log K_d values were calculated as described in the materials and methods section. All pressures were measured with the vapour pressure osmometer (model 5500 XRS: Wescor). These values are the averages of independent experiments with the numbers of trials as follows: PEG MW 10 000, n=3; PEG MW 8 000, n=3; PEG MW 6000, n=3; PEG MW 3000, n=3; PEG MW 2000, n=4; PEG MW 1500, n=5; PEG MW 1000, n=2; PEG MW 600, n=2; PEG MW 400, n=2; PEG MW 300, n=2. All values are contained in Appendix Table VII.


0.75
0.5
0.25
0
log $K_d°/K_d^{\Pi}$ (mmolal)
0
1
2
3
4
5
Π (dynes/cm²*10⁷)
PEG 10 000
PEG 8000
PEG 6000
PEG 3000
PEG 2000
PEG 1500
PEG 1000
PEG 600
PEG 400
PEG 300

Appendix Table VII). Figure 22 shows representative examples of these results to highlight specific trends. Three trends are observed.

The first trend is that the glucose affinity of hexokinase increases as a function of increasing osmotic pressure. These increases are observed in the presence of all PEGs, regardless of the molecular weight.

The second trend is that in the presence of all the different molecular weight PEGs the relationship between the log glucose affinity ratio and osmotic pressure tends to be nonlinear. However, in the presence of PEGs less than molecular weight 1500, the relationship between the log glucose affinity ratio is more linear than in the presence of PEGs greater than MW 1000 (regression coefficients in Appendix Table VIII). The regression coefficients calculated for all polymers below MW 1500 are above 0.9.

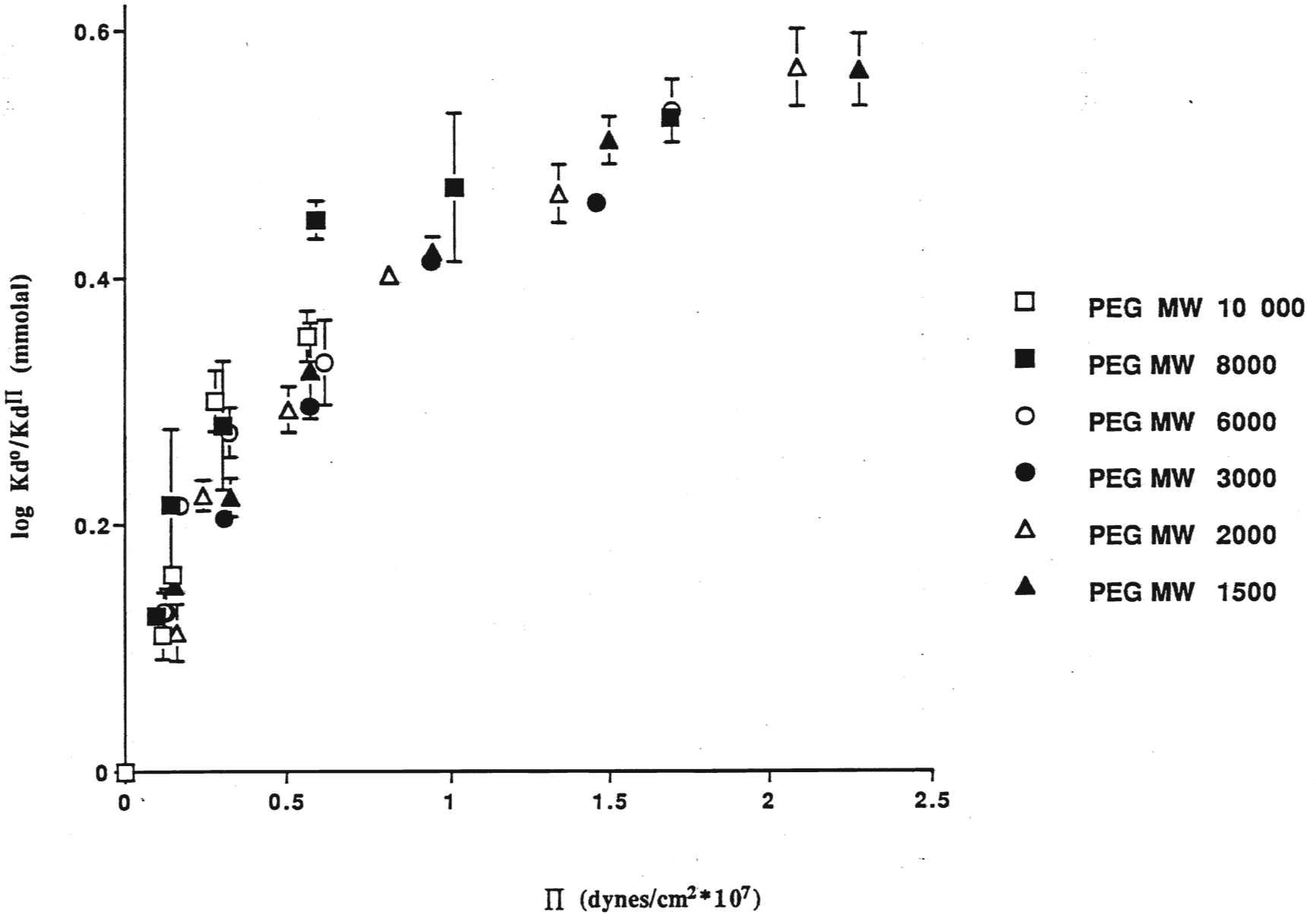
The third trend is that the log glucose affinity ratios in the presence of PEGs lower than MW 1500 are systematically less than those in the presence of PEGs above MW 1500. The standard errors of the means for the log affinity ratios vs. pressure, calculated in the presence PEG MW 10000, 8000, 6000, 3000, 2000, and 1500, are seen in figure 23. The results show that differences in the log glucose affinity ratios calculated in solutions containing PEGs of higher molecular weight than 1500, are not larger than one standard error of the mean.

In the presence of PEGs lower than MW 1500, the results show decreased log glucose affinity ratio values, at equivalent osmotic pressures. For example, at 2.1 x 10^7 dynes/cm^2 the average log glucose affinity ratio calculated in the presence of PEG MW 1500 is 42% higher than the average observed in solutions containing PEG MW 300. This

Figure 22. Representative examples to clearly illustrate the relationships between log $K_d°/K_d^{\Pi}$ verses osmotic pressure in the presence of PEG MW 8000, 600, 300. All pressures were measured with the vapour pressure osmometer (model 5500 XRS: Wescor). These values are the averages of independent experiments with the numbers of trials as follows: PEG MW 8000, n=3; PEG MW 600, n=2; PEG MW 300, n=2.

PEG 1500
PEG 600
PEG 300
Log K_d^o/K_d^Π (mmolal)
0.6
0.4
0.2
0
0
1
2
3
4
5
Π (dynes/cm^2*10^7)

Figure 23. The standard errors of the means for the relationships between log K_d°/K_d^{Π} verses osmotic pressure in the presence of PEG MWs 10 000 (n=3), 6000 (n=3), 3000 (n=3), 2000 (n=4), and 1500 (n=5).


log Kd°/Kd^Π (mmolal)
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
Π (dynes/cm²*10⁷)
PEG MW 10 000
PEG MW 8000
PEG MW 6000
PEG MW 3000
PEG MW 2000
PEG MW 1500

shows that under equivalent osmotic pressures, hexokinase has less affinity for glucose in the presence of PEGs lower than MW 1500, as compared to higher molecular weight PEGs. The linearity of the relationships between the log glucose affinity ratios and osmotic pressure allows statistical tests to determine if the slopes of the lines are statistically different. The calculation of the difference between the slopes are given in Appendix II.

The results in Appendix Table IX display statistical t-test values and show that at, or below, the 0.05 level of significance, the slopes of all lines are significantly different, with exception to the slopes for PEG MW 400 and 300. These results provide statistical evidence for a difference between the relationship between the calculated log glucose affinity ratios and osmotic pressure, in the presence of these different molecular weight PEGs.

The Changes in the Number of Polymer-Inaccessible Water Molecules Upon the Binding of Hexokinase to Glucose.

According to the osmotic stress theory, changes in the GEDC of hexokinase under osmotic pressure can be interpreted as the result of changes in hydration. The relationship between the log glucose affinity ratio and osmotic pressure allows the calculation of ΔN_w (equation 6). These volumes of water have been converted into numbers of water molecules by dividing by the molecular volume of one water molecule, as shown in figure 24. Figure 25 shows representative examples to highlight the trends in the values of ΔN_w in the presence of various molecular weight polymers (values are contained in Appendix Table X).

The results show three main trends with respect to the values of ΔN_w: 1) ΔN_w decreases as a function of increasing osmotic pressure, 2) in the presence of polymers below MW 1500, there is less of a change

Figure 24. The relationship between the change in ΔN_w with respect to the osmotic pressures of various molecular weight PEG solutions. The change in the volume of polymer-inaccessible water(ΔV_w) upon the binding of glucose to hexokinase is determined from equation 6. By plotting the log glucose affinity ratios with respect to osmotic pressure, the slope of the line of best fit allows the calculation of ΔV_w upon the binding of glucose to hexokinase. Since the relationship is curvilinear in the presence of many molecular weight PEGs, all the $\log K_d^{\circ}/K_d^{\Pi}$ verses osmotic pressure data for each MW PEG were plotted simultaneously and lines of best fit were drawn by eye through these data points. Tangents to these curves were then estimated graphically in order to obtain the slopes of the tangents to the curves at the measured osmotic pressures. The tangents to these curves allowed the calculation of the changes in ΔV_w upon the binding of glucose to hexokinase. The change in ΔN_w was calculated by dividing ΔV_w by the volume of one water molecule. All values are contained in Appendix Table X.

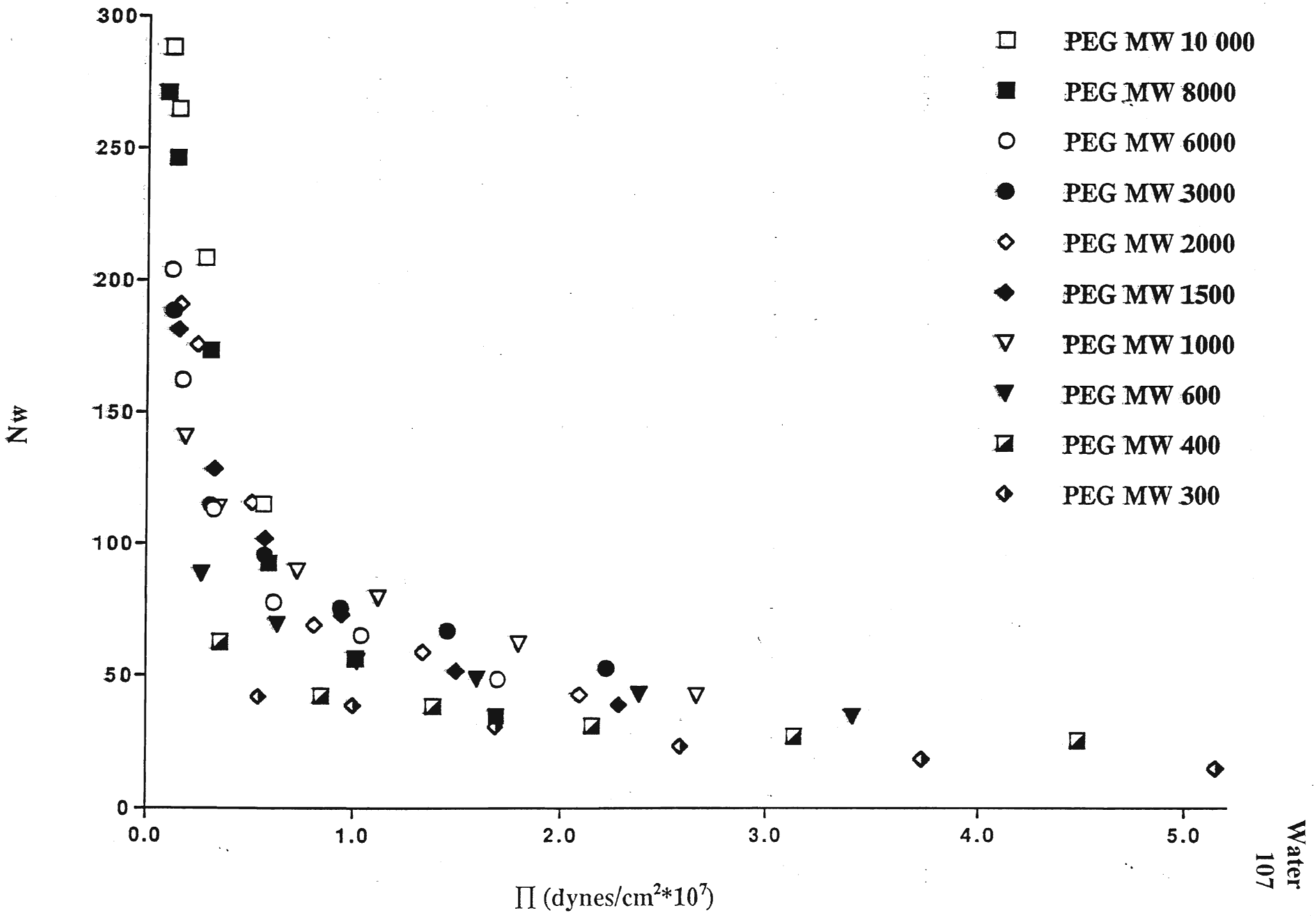
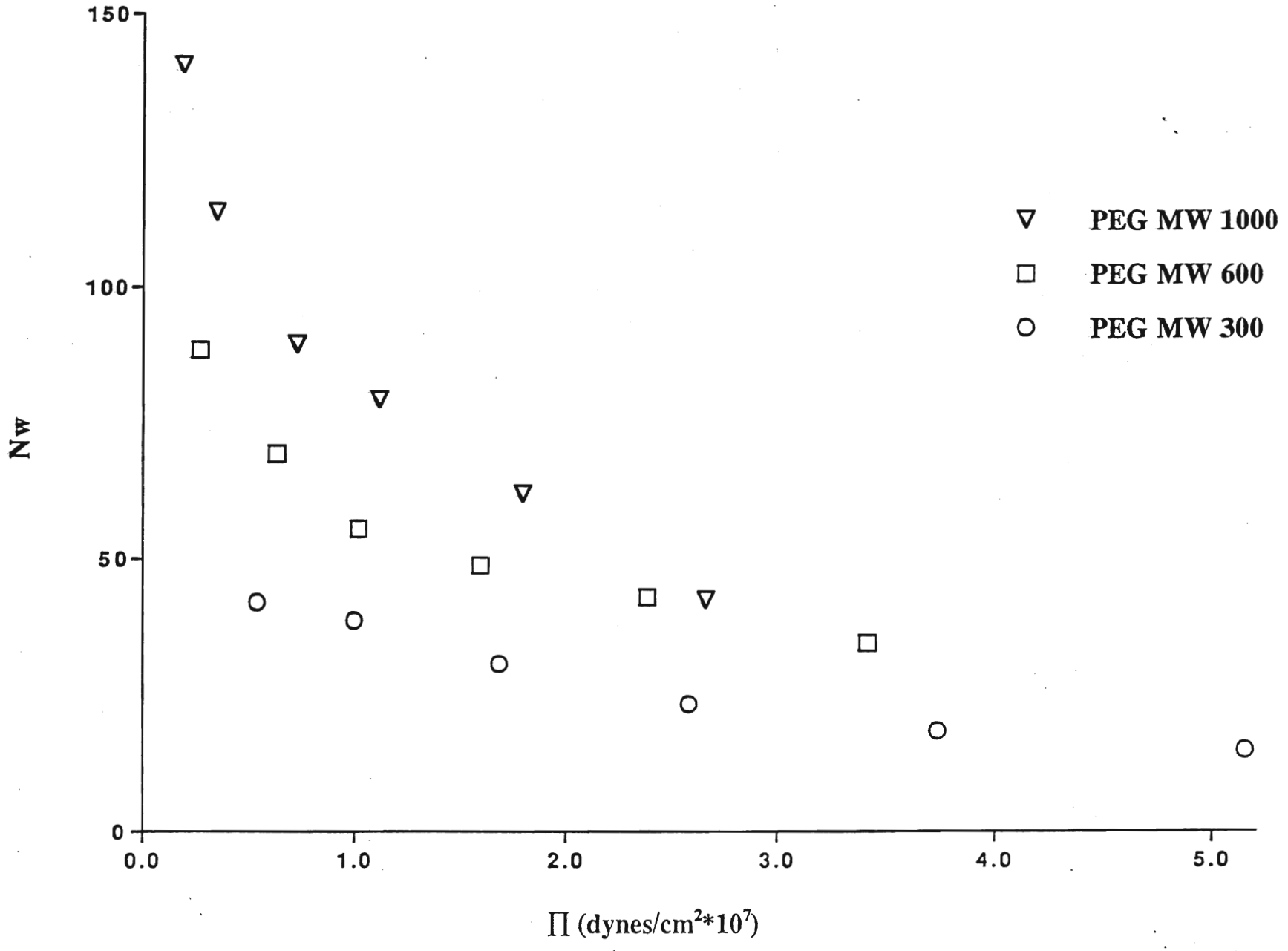

300
250
200
150
100
50
0
Nw
0.0
1.0
2.0
3.0
4.0
5.0
Π (dynes/cm²*10⁷)
PEG MW 10 000
PEG MW 8000
PEG MW 6000
PEG MW 3000
PEG MW 2000
PEG MW 1500
PEG MW 1000
PEG MW 600
PEG MW 400
PEG MW 300

Figure 25. Representative examples of the changes in ΔN_w as a function of osmotic pressure in the presence of PEG MW 1500, 600, and 300.


150
100
50
0
Nw
0.0
1.0
2.0
3.0
4.0
5.0
Π (dynes/cm²*10⁷)
PEG MW 1000
PEG MW 600
PEG MW 300

in the values of ΔN_w with osmotic pressure than in the presence of PEGs higher than MW 1000, and 3) at equivalent osmotic pressures, there is a large amount of scatter in the change in the values for ΔN_w in the presence of polymers higher than MW 1000.

It may be clearly seen from figures 24 and 25 that ΔN_w (proportional to the slopes of tangents to these curves) decreases as osmotic pressure increases. For example, in the presence of PEG MW 10000, as many as 290 polymer-inaccessible water molecules are liberated upon hexokinase binding to glucose at low pressure (below 1 x 10^7 dynes/cm^2), but this value is reduced to approximately 15 at high pressure. However, these results are affected by the high sensitivity of hexokinase to low osmotic pressures and the presence of the different molecular weight polymers.

The results show that at low osmotic pressures (below 1 x 10^7 dynes/cm^2) there are wide ranges in the values of ΔN_w in the presence of the various molecular weight PEGs. For example, in the presence of the different molecular weight polymers the calculated changes in the numbers of polymer-inaccessible water molecules range between 45-290 (below 1 x 10^7 dynes/cm^2). However, measurements show that at twice this osmotic pressure, ΔN_w is reduced to between 30-60, in the presence of the various PEGs. These results show the sensitivity of ΔN_w at low osmotic pressures.

The results in figures 24 and 25 show that the molecular weight of the polymers used in the stressing solutions affects ΔN_w. At essentially equivalent pressures, there is less of a change in the values of ΔN_w in the presence polymers lower than molecular weight 1000 (Figs. 24 and 25). This is mainly observed below the osmotic pressure of 1 x 10^7 dynes/cm^2.

For example, below 1 x 10^7 dynes/cm^2 ΔN_w, ranges between 145 to 290 in the presence of polymers between the molecular weights of 1000 and 10000. These differences decrease with increasing osmotic pressure until an apparent limiting value of approximately 25 water molecules is reached.

Discussion.

The Osmotic pressures of Polymer Solutions Measured by Vapour Pressure Osmometry.

The measured osmotic pressures of the polymer solutions increase with polymer concentration, but deviate from ideality. The measurements of solutions containing PEG MW 6000 agree with other published measurements (Michel, 1983) to within five percent. Solutions containing higher molecular weight PEGs show greater osmotic pressure non-ideality (Zimmerman et al., 1993). PEG solution non-ideality increases with: 1) increases in the molecular weight of the dissolved PEG and 2) increases in PEG concentration (Zimmerman et al., 1993). It is because of the non-ideality of the osmotic pressures of PEG solutions that measured osmotic pressures are used in the present experimental study of "osmotic stress." These measurements form an osmotic pressure data base for all future work.

Non-additivity in the Osmotic Pressures of Solutions Containing Polyethylene Glycol.

The results of the present investigation indicate that the osmotic pressures of PEG and co-solute solutions including glucose are non-additive, i.e.. that the osmolality of glucose appears to be higher in the presence of PEG than in its absence. This trend results from decreases in the quantity of water that is available to glucose in the presence of PEG. Previous investigations have also documented PEG dependent increases in the concentrations of co-solutes.

Michel, (1973, 1983), used vapour pressure osmometry techniques to observe that PEG MW 6000 concentrates several co-solutes in solution. Bezrukov and Vodyanoy (1993) measured increases in the concentration of sodium ions with a sodium electrode. They observed

that the concentration of sodium ions increased with increases in PEG MW 400 concentration (Bezrukov & Vodyanoy, 1993). Interestingly, not all polymers increase the concentrations of co-solutes in solution.

Bezrukov and Vodyanoy (1993) have shown that dextran does not increase the concentration of the co-solute sodium chloride in solution. However, dextran has been found to interact with hexokinase, which eliminates it as an osmoticant in the present investigation (Rand et al., 1993). Otherwise, dextran would simplify the interpretation of the GEDC data collected for hexokinase. The study of the PEG osmotic pressure non-additivity with co-solutes reveals some interesting facts about this polymer.

Appendix Table I displays the gms. glucose-inaccessible water per gm. PEG (the ratios showing the moles of glucose-inaccessible water/mole of PEG are contained in Appendix Table II). These ratios show that the quantities of glucose-inaccessible water range between 1.53 to 0.77 g water/g PEG. Zimmerman et al. (1993) use a graphical method to calculate the quantity of solvent per solute in solution to estimate non-ideality. These ratios have values ranging between 3.67 to 0.12 g water/g PEG. The calculations give ratios that are generally a factor of two higher than those observed within the present investigation. It is probable that the discrepancies result from differences in the methods used to define the PEG/water interaction.

The quantities of glucose-inaccessible water in the presence of the different molecular weight PEGs show three main trends: 1) for all molecular weight PEGs, the quantity of glucose-inaccessible water decreases with increases in PEG concentration; and 2) the rate at which the quantity of glucose-inaccessible water decreases is higher

in the presence of higher molecular weight PEGs (a representative example can be seen in Fig. 26).

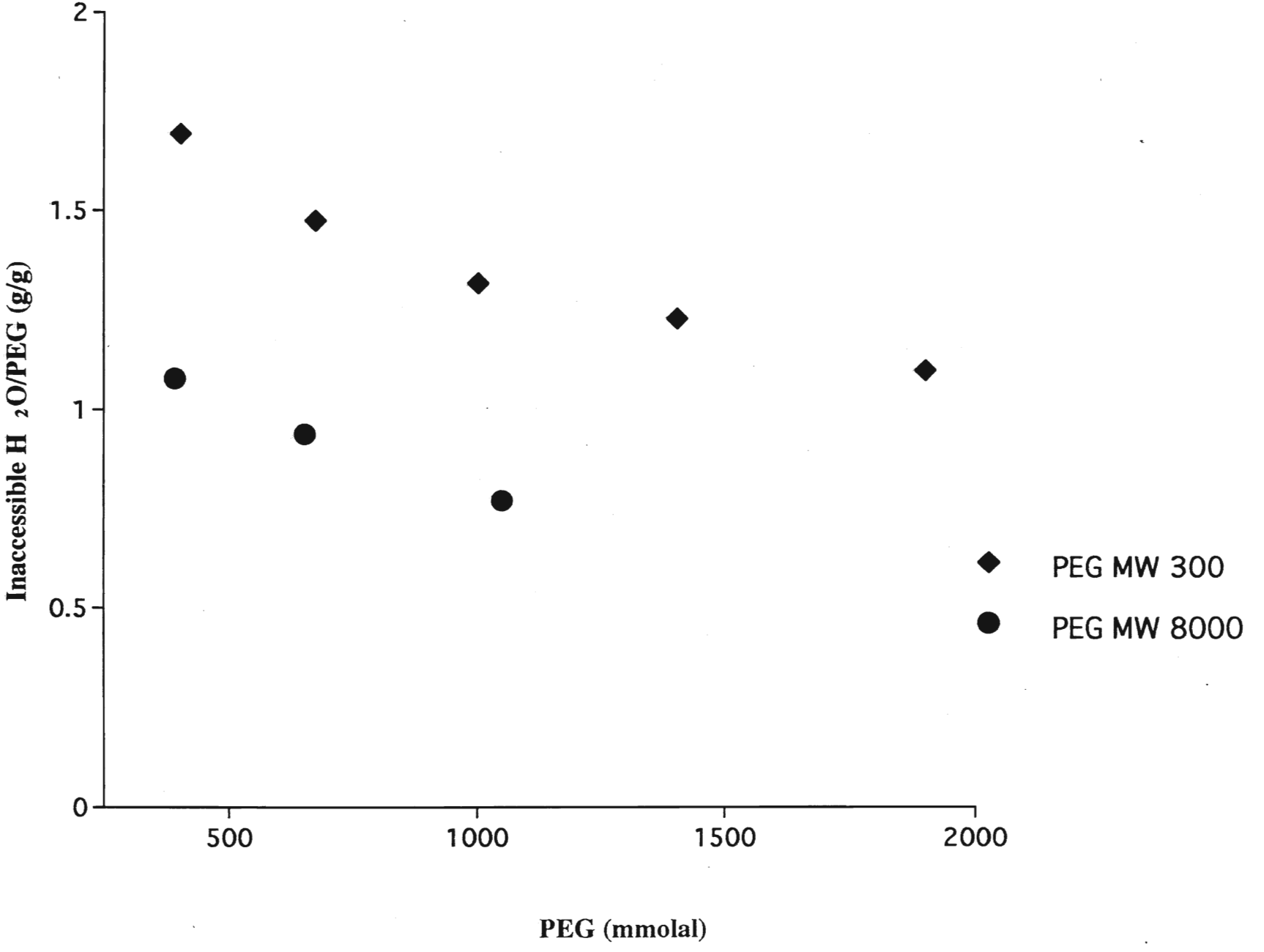
The first trend is that the quantity of glucose-inaccessible water decreases with increases in polymer concentration. Since the polymers are hydrated by a certain quantity of water, and the proportion of water within the solutions decreases with increases in polymer concentration, then it would be reasonable to propose that there will be less water accessible to both co-solutes as the PEG concentration increases. Therefore, as the concentration of the polymer increases, it would be expected that the quantity of water available to hydrate both the polymer and glucose will decrease.

The second trend is that there are higher rates at which the quantity of glucose-inaccessible water decreases in the presence of higher molecular weight PEGs. This finding can partially be interpreted using the non-ideality of the solutions containing higher molecular weight polymers. The non-ideality of polymer solutions increases with increases in both the molecular weight of the polymers and their concentration (Zimmerman et al., 1993). Therefore, the non-ideality for the higher molecular weight polymer solutions would be greater as the concentration of the polymer increases.

Comparison of Polymer Solution Osmotic Pressures Measured by Vapour Pressure Osmometry and Secondary Osmometry Using Phospholipids.

Comparison of the results obtained by the VPO and secondary osmometry with phospholipids shows two trends: 1) equivalent osmotic pressures are measured for solutions containing PEG MW 1500 and 1000 and 2) secondary osmometry measures lower osmotic pressures for solutions containing less than 30 weight percent PEG MW 400 and 300.

Figure 26. Representative example of the differences in the rate at which the amount of water/PEG (g/g) with respect to molality is higher in the presence of PEG MW 8000 than PEG MW 300.


2
1.5
1
0.5
0
500
1000
1500
2000
Inaccessible H 2O/PEG (g/g)
PEG (mmolal)
PEG MW 300
PEG MW 8000

The similarities and differences will be discussed with reference to the principles of both osmometry techniques.

The VPO used in the present investigation measures changes in the dew point of a solution to determine its ideal osmotic pressure (Unidentified, Wescor). This method has distinct advantages over other osmometry methods because it does not require the partitioning of the polymer molecules by any physical membrane.

Measuring osmotic pressure by secondary osmometry using phospholipids is based upon the size dependent exclusion of polymer molecules from between the phospholipid bilayers (Parsegian et al., 1986). If a polymer molecule is not excluded from between the bilayers, it will not contribute to the osmotic pressure measured by this technique (Parsegian et al., 1986). Furthermore, if a certain proportion of solute molecules has access to the inter-bilayer space, then the osmotic pressure measured by this technique will be incorrectly low (Parsegian et al., 1986). The results show that the osmotic pressure measurements of solutions containing less than 30 weight percent PEG MW 400 and 300 are lower than those measured with the VPO. These findings provide evidence that a proportion of these low molecular weight polymers are not excluded from in-between the phospholipid bilayers.

The results also show that the phospholipid and vapour pressure osmometry techniques measure equivalent osmotic pressures for solutions containing 30 weight percent PEG MW 400 and 300. These findings indicate that the polymer molecules are completely excluded from between the phospholipid bilayers in these solutions. The complete exclusion of the low molecular weight polymers in the 30

weight percent solutions may be interpreted with respect to changes in the inter-bilayer spacings under osmotic pressure.

Secondary osmometry with phospholipids uses inter-bilayer d-spacings of multi-lamellar phase phospholipids as a yardstick by which solutions of unknown osmotic pressure are measured (LeNeveu et al., 1977). Previous investigations have revealed that the d-spacings between bilayers decrease under osmotic pressure (LeNeveu et al., 1977). Since polymer molecules are excluded from in-between the bilayers on the basis of their size (Parsegian et al., 1986), it is reasonable to propose that as the inter-bilayer space decreases under osmotic pressure, a greater proportion of the low molecular weight polymer molecules will be excluded. The results of the present investigation show that under the log osmotic pressure of 30 weight percent solutions of PEG MW 400 and 300 (7.7 dynes/cm^2) the d-spacings (55-56 Å) are small enough to totally exclude both PEG MW 400 and 300.

The Effects of Salt and pH on the Glucose Equilibrium Dissociation Constant.

The results of the present investigation indicate that the GEDC of hexokinase is insensitive to pH and has a low sensitivity to ionic strength, within the ranges investigated. Similar insensitivity has been observed in the apparent glucose dissociation constant of hexokinase under high ionic strength (Feldman & Kramp, 1978) and pH conditions (Feldman & Kramp, 1978; Hoggett & Kellett, 1976). The findings of the present investigation show that PEGs decrease the GEDC of hexokinase by as much as a factor of ten. We conclude that any PEG dependent change in pH or potassium chloride concentration cannot

account for the observed decreases in the GEDC of hexokinase in the polymer solutions.

Altekar (1977) investigated the effects that increased salt concentrations have on decreases in the intrinsic fluorescence of several proteins. His investigation has shown concentration dependent decreases in the intrinsic tryptophan fluorescence of several proteins that are specific to the salt used. Potassium chloride induced the smallest changes in intrinsic fluorescence of all salts studied, and these effects were only observed above the concentration of 2 molar (Altekar, 1977). The results of Altekar (1977), and those obtained with hexokinase in the present investigation, indicate that potassium chloride has little effect on the intrinsic fluorescence of hexokinase.

The Relationship Between the log Glucose Affinity Ratios and Osmotic Pressure.

The relationship between osmotic pressure and the log glucose affinity ratios of hexokinase in the presence of the different molecular weight PEGs show three general trends: 1) the log glucose affinity ratios of hexokinase increase with osmotic pressure; 2) for all the PEGs there is a common curvilinear relationship between the log glucose affinity ratios and osmotic pressure, however, in the presence of PEGs greater than MW 1000, the curvilinear relationship is more pronounced; and 3) in the presence of PEGs less than MW 1500 the log glucose affinity ratios systematically decrease with the molecular weight of the PEG, at equivalent osmotic pressures.

The first trend shows that the log glucose affinity ratios of hexokinase increase as a function of osmotic pressure. This shows that under osmotic pressure the equilibrium between glucose bound and dissociated conformations shifts in favour of the glucose bound

conformation. These results agree with those obtained by Rand et al. (1993), who previously observed this trend. The observed differences between the GEDC values obtained in the present investigation and those obtained by Rand et al. (1993) can be attributed to improvements in experimental protocols.

The second trend is that there are curvilinear relationships between the log glucose affinity ratios and osmotic pressure in the presence of the PEGs. The curvilinear relationship shows that the GEDC becomes less sensitive to osmotic pressure as the osmotic pressure applied to hexokinase increases.

For example, if the GEDC was equally sensitive to increases in osmotic pressure over the entire range investigated, the relationship between the log glucose affinity ratio and osmotic pressure would be linear. However, the results show the effect that osmotic pressure has on glucose affinity is not constant, but decreases with osmotic pressure. This curvilinear relationship was not detected in the results of Rand et al. (1993) due to the scatter of their data.

The third trend is that the log glucose affinity ratios are higher in the presence of PEGs with molecular weights greater than 1000. Under the conditions of the experiments, the PEG solutions were prepared by wt% concentrations so they are not of the same molality, and do not have the same osmotic pressures. This precludes statistical testing for differences in the mean log glucose affinity ratios measured in the presence of the various molecular weight PEGs. However, by extrapolation of the relationship between these variables it is discovered that in the presence of PEGs greater than MW 1000, all calculated values fall within one standard error of the mean.

The results also show that in the presence of polymers less than MW 1500, the relationship between the log glucose affinity ratios and osmotic pressure, have linear regression coefficients greater than 0.90. Significance testing rejects the null hypothesis that the slopes of the regression equations come from the same population slope (at the 0.05 probability level), except for PEG MW 400 and 300. The results show that there are statistically significant differences between the slopes of the log glucose affinity ratios and osmotic pressure in the presence of the majority of the low molecular weight polymers.

The Calculated Changes in the Number of Polymer-Inaccessible Water Molecules Upon the Binding of Glucose Binding to Hexokinase.

ΔN_w is defined as the difference in the number of water molecules that are polymer-inaccessible between the two conformations of hexokinase. The calculation of the change in water is determined by measuring the change in the glucose affinity of hexokinase with respect to osmotic pressure (equation 6). Since the change in the glucose affinity of hexokinase with respect to osmotic pressure is not constant, curvilinear relationships between these variables are observed. Determining the slopes of the tangents to these curves at the osmotic pressures applied in the experiments allows the calculation of the change in ΔN_w. These values show several trends.

The results also show that the relationship between the log glucose affinity ratios and osmotic pressure in the presence of polymers less than MW 1500, tend to be more linear than in the presence of PEG greater than MW 1000. This indicates that the change in ΔN_w remains relatively constant with osmotic pressure range investigated.

The results show three main trends with respect to the change in ΔN_w upon the binding of glucose to hexokinase: 1) ΔN_w decreases as a function of increasing osmotic pressure; 2) at equivalent osmotic pressures, there is a large scatter in the values representing the change in ΔN_w in the presence of polymers higher than MW 1000; and 3) there is a smaller change in ΔN_w in the presence of polymers below MW 1500, extrapolated to zero osmotic pressures.

The first trend shows that as the osmotic pressure of the environment surrounding hexokinase increases, the difference in the volume of polymer-inaccessible water between conformations decreases. This observed decrease in ΔN_w differs from the results obtained by Rand et al. (1993), because they did not observe the curvilinear relationships between the log glucose affinity ratios and osmotic pressure.

By extrapolating from the lowest pressure applied to zero pressure, it is determined that the numbers of polymer-inaccessible water molecules increases with PEG molecular weight, up to MW 1500. Thereafter, the changes in ΔN_w tends to be constant, but shows large scatter.

The second trend shows that there is greater scatter in the values for the changes in ΔN_w at lower, as opposed to higher, osmotic pressures. This result may be interpreted as the result of the high sensitivity of the calculation to the initial relationship between the log glucose affinity ratios and osmotic pressure. At low pressures, in the presence of the various molecular weight PEGs, these numbers range between 45-290. At the highest pressure used in this investigation this value reaches 15. These values reflect greater changes in the values of ΔN_w are higher than the 65 $\pm$ 10 water

molecules reported by Rand et al. (1993). The differences between the calculated values obtained in both investigations are likely due to modifications in the experimental protocol, and a wider range of molecular weight PEGs, used within the present investigation.

The third trend is that the values of ΔN_w are lower in the presence of the lower molecular weight polymers. Two possible mechanisms by which this could occur are explained later in the discussion.

What Water is Measured by the Osmotic Stress Technique?

Before further discussion of ΔN_w, it is important to stress what water molecules are sensitive to osmotic stress. The measured waters are: 1) those that are involved in the conformational change of the enzyme, and 2) are inaccessible to PEG. If the polymer molecules have access to a proportion of the water that contributes thermodynamically to the conformational change of hexokinase, that water will not be measured by this technique.

Interpretation of Hydration Changes with Respect to the Interactions Between PEG and Proteins.

In discussing the results of the present investigation, it is important to consider the relationship between PEGs and proteins in solution. The application of the osmotic stress technique relies on the exclusion of PEG from a volume of water surrounding hexokinase. Timasheff and his coworkers have shown that PEG is excluded from the domains of several proteins (Arakawa & Timasheff, 1985 a,b; Bhat & Timasheff, 1992; Lee & Lee, 1987). The process by which this occurs is summarized by Timasheff (1992). It is also important to determine the factors that change the volume of exclusion of PEG from the domain of hexokinase.

It has been determined that the volumes of exclusion (V_e) of several molecular weight PEGs from the domains of several proteins decreases with: 1) increases in PEG concentration (Arakawa & Timasheff, 1985 a,b; Bhat & Timasheff, 1992) and 2) decreases in PEG molecular weight (Bhat & Timasheff, 1993). The volume of exclusion (V_e) is defined as the volume of the water shell that surrounds the protein from which the centers of mass (radii of exclusion, (R_e) of the polymers are excluded (Schachman & Lauffer, 1949). It is possible to interpret the changes in ΔN_w due to osmotic pressure with reference to the exclusion of the polymer molecules from the domain of hexokinase.

The results show that under increased osmotic pressure, ΔN_w decreases. Within the present investigation, higher osmotic pressures are applied to hexokinase by exposing it to higher PEG concentrations. Therefore, the observed decrease in ΔN_w might be interpreted as the result of decreases in the PEG excluded volumes of water around hexokinase.

For example, if PEG at higher concentrations has access to greater proportions of the water that contributes thermodynamically to the conformational change of hexokinase, the amount of water measured by the osmotic stress technique will decrease. This interpretation explains why under higher osmotic pressures ΔN_w decreases. However, this does not explain the results showing the molecular weight dependence on ΔN_w.

Bhat and Timasheff (1990) show that the exclusion of PEG from the domains of several proteins increases with the molecular weight of PEG. Changes in the volumes of polymer-inaccessible water liberated do not show a clear trend with respect to polymers above MW 1000. It is probable that the large calculated volumes of polymer-inaccessible

water liberated in the presence of the higher molecular weight polymers make any molecular weight dependent changes difficult to detect.

In the presence of polymers less than MW 1500, the results show that ΔN_w systematically decreases with PEG molecular weight. These findings are consistent with the decreases in the excluded volume of PEG molecules from around proteins proposed by Bhat and Timasheff (1993). The smaller ΔN_w values may highlight the molecular weight dependence that is not observed in the presence of the higher molecular weight PEGs.

It is also possible to interpret decreases in ΔN_w in terms of the geometry of hexokinase. The results indicate that polymers lower than MW 1500 show systematically decreased ΔN_w values. Since PEG size depends upon molecular mass (Mark & Flory, 1965), smaller molecular weight polymers may have access to aqueous compartments around hexokinase that exclude the larger polymers. This interpretation explains the sudden molecular weight dependence observed for polymers below MW 1500, and why the wide range of higher molecular weight polymers do not affect the quantity of polymer-inaccessible water liberated.

Modeling the Conformational Change of Hexokinase.

The osmotic stress measurements show that at low osmotic pressure, ΔN_w is approximately three hundred. With the aid of computer models (Quanta, Molecular Simulations, Waltham, MA), it was possible to determine the difference in the number of water molecules that could fit around the enzyme in both open and closed conformations (Molecular Simulations Incorporated). By calculating the differences in the solvent accessible Connolly surfaces (Connolly, 1983) between open

and closed conformations it is determined that the open conformation contains 57 more water molecules than the closed conformation. The differences between the open and closed conformations out to two hydration shells, are 77 and 134 water molecules, respectively. One outstanding problem to be solved is why there is such a large discrepancy in the differences in the number of water molecules associated with the open and closed conformations of hexokinase as determined by the modeling and the osmotic stress technique.

One possible explanation for the discrepancy is that the water that is thermodynamically significant to the conformational change of hexokinase extends to more than two hydration layers around hexokinase. Bhat and Timasheff (1992) indicate that the volume of exclusion from the domain of several proteins for PEG MW 200 to range between 1-6 Å, and for PEG MW 6000 to be between 26-41 Å. These excluded volumes would consist of several hydration shells. However, it must be emphasized that osmotic stress measurements give no insight as to the location of the water molecules (Parsegian et al., 1986). It is also possible that it is inappropriate to attempt to match the crystal structure data to the data obtained from the enzyme in solution.

The process of crystallization can impart order upon the protein structure that does not occur in free solution (Voet & Voet, 1990). This suggests that the structures of the crystallized and free proteins may be different, and that comparison of solution and crystal hydration during the conformational change of hexokinase may be inappropriate.

The results obtained within this investigation show that at higher osmotic pressures, the change in the volume of polymer-inaccessible water molecules upon the binding of glucose to hexokinase

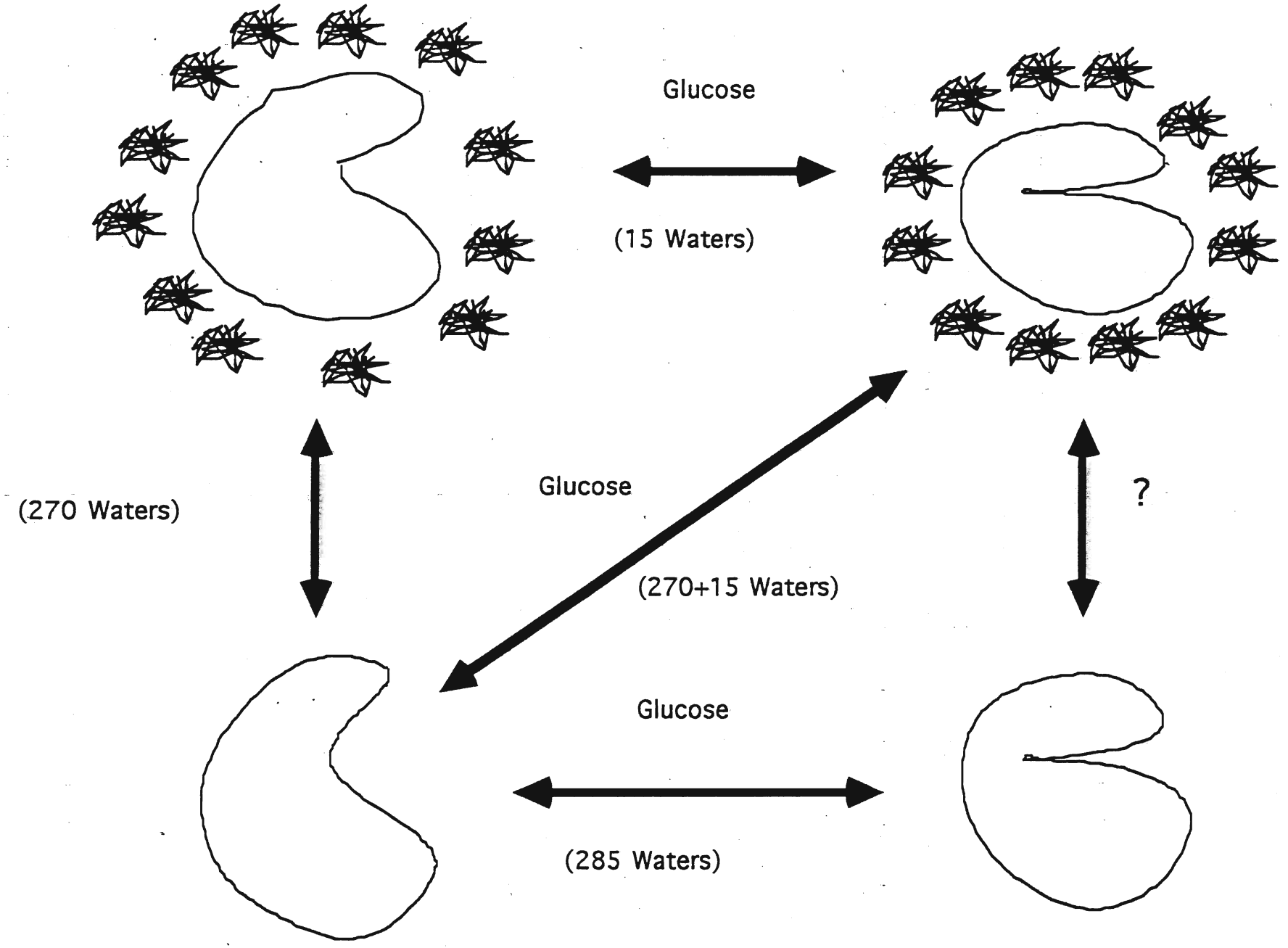
decreases. The change in the numbers of waters at high PEG concentration, a well known crystallization agent (McPherson, 1985), more closely agree with the calculations obtained from the crystal structures. These findings suggest that the structure of hexokinase in free solution may be more hydrated that the crystal structure of the enzyme, and that the addition of the polymers tend to shift the solution structure closer to that of the crystal structure. A simple model has been developed that attempts to reconcile the observed effects of osmotic pressure and PEG molecular weight to hexokinase.

The proposed model may be seen in figure 27 and relates the effects of osmotic pressure to changes in ΔN_w. This model hypothesizes that both the glucose bound and the free enzyme can exist in an continuum of osmotically stressed states that change depending on the applied osmotic pressure. The quantity of polymer-inaccessible water liberated upon glucose binding is dependent upon the osmotically stressed state which hexokinase is in.

The binding of glucose to the unstressed enzyme is associated with the liberation of approximately 295 polymer-inaccessible water molecules. Subsequent to the application of osmotic pressure, the numbers of liberated water molecules are reduced. At the maximum pressure applied in this investigation, the binding of glucose corresponds to the liberation of only 25 water molecules. Therefore, the application of osmotic pressure must decrease the number of polymer-inaccessible water molecules by 270.

Two plausible mechanisms may act separately, or cooperatively, to account for the decrease of 270 polymer-inaccessible water molecules that are liberated under osmotic pressure. These are: 1) there is a change in the exclusion of PEG from water that is significant

Figure 27. A hypothetical model to explain how the changes in ΔN_w are affected by both the access of the polymer molecules to the water that is thermodynamically significant to the conformational change of hexokinase and the effects of osmotic pressure on the conformation of hexokinase. Two hypotheses are apparent: 1) the access of the polymer molecules to the water that is thermodynamically significant to the conformational change increases with increases in polymer concentration, and decreases in polymer molecular weight or; 2) there are changes in the conformation of hexokinase as the result of applied osmotic pressure which will result in decreases in the change in ΔN_w. The question mark on the figure denotes a conformational change that can not be observed with the methods used within the present investigation.


Glucose
(15 Waters)
(270 Waters)
Glucose
(270+15 Waters)
?
Glucose
(285 Waters)

to the conformational change of hexokinase, and 2) there is a change in the geometry of hexokinase under osmotic pressure to forms more closely represented by the crystal structures. Both of these mechanisms have previously been discussed with respect to the hydration of hexokinase.

The question mark in figure 27 denotes a possible change in the hydration of hexokinase for which the experimental techniques used within this investigation do not give any information. In order for the osmotic stress technique to measure changes in the hydration of enzymes, there has to be a signal that is altered by the application of osmotic pressure.

Suggested Further Experiments.

The results obtained within the present investigation show that the equilibrium binding of glucose to hexokinase is a good system for the application of osmotic stress measurements. This makes this system a prime candidate for further investigation. To increase the robustness of the results, it would be beneficial to use polymers, other than PEG, to apply osmotic pressure. If equivalent osmotic pressure associated with chemically different polymers produced the same effect on the GEDC of hexokinase, this would provide further evidence that the effects obtained within this investigation were due solely to changes in water activity.

It would also be beneficial to determine how the affinity of other substrates would change due to osmotic pressure, specifically how these substrates affect the changes in ΔN_w. Hypothetically, low affinity substrates should be associated with similar changes in the volume of polymer-inaccessible water upon substrate binding. Furthermore, substrates that influence the conformational change of

hexokinase, in ways different than glucose, should change the quantities of polymer-inaccessible water liberated upon substrate binding.

A very important extension of the present research lies in determining why there are such large volumes of polymer-inaccessible water liberated upon hexokinase binding to glucose. As previously discussed, the results suggest that the conformation of hexokinase in free solution may be different than in the crystal structure. Methods such as Fourier Transform Infrared Spectroscopy may allow the measurement of changes in the structure of hexokinase under osmotic pressure.

Conclusions.

The study of the forces that mediate the association of structures is of fundamental importance to biology. The osmotic stress technique has been developed in order to investigate these forces, and its power lies in its broad applicability. This technique has been used to show that hydration forces are not only important in maintaining molecular association (e.g. lipid bilayers), but also affect the conformations of macromolecules.

Within this investigation the osmotic pressures of the polymers solutions were measure by both vapour pressure osmometry and secondary osmometry with phospholipids. The results have pointed out similarities and differences in the osmotic pressure values obtained with both methods. Vapour pressure osmometry has also allowed the discovery that PEG tends to concentrate co-solutes in solution. The concentrating effects are discussed with reference to the non-additivity of osmotic pressures, as well as the quantity of water associated with the polymers.

The results obtained indicate that osmotic pressure increases the glucose affinity of hexokinase. These changes are complex and may be interpreted in terms of the exclusion of PEG from around hexokinase, as well as PEG induced changes in the geometry of the enzyme.

Summary of Key Findings.

1) Both the VPO and secondary osmometry with phospholipids give equivalent measurements of osmotic pressures produced by solutions of PEG MW 1500 and 1000, but the phospholipid technique is not suited for the osmotic pressure measurements of dilute solutions containing PEG MW 400 and 300.

2) The osmotic pressures of co-solute solutions containing glucose and PEG are non-additive. This shows that the sum of the osmotic pressures measured for single solute solutions containing either glucose or PEG are less than the osmotic pressure of the co-solute solution. The results show that the effective concentration of the co-solute increases in a PEG concentration dependent manner.

3) The Glucose Equilibrium Dissociation Constant of hexokinase is insensitive to changes in pH and potassium chloride concentrations within the ranges investigated.

4) The development of a new procedure reduced in the scatter of the GEDC measurements and allowed: A) a better resolution of the change in the glucose affinity of hexokinase with osmotic pressure, and B) more precise measurements of the volumes of polymer-inaccessible water liberated as the result of glucose binding to hexokinase.

5) The glucose affinity of hexokinase increases as a function of the applied osmotic pressure.

6) The sensitivity of hexokinase's glucose affinity decreases with increasing osmotic pressure in the presence of PEGs greater than MW 1000.

7) Lower molecular weight polymers have a smaller effect on the glucose affinity of hexokinase, as compared to the higher molecular weight polymers, at the same osmotic pressure.

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Appendix I.

Derivation of Osmotic Pressures from the Vapor Pressure Osmometer.

From Raoult's law, the vapor pressure of a solution may be related to the concentration of solute in it by the following:

$$P = \frac{n_W P^\circ}{n_S + n_W} \quad (14)$$

where n_w is the number of moles of water and n_s is the number of moles of solute. The number of moles of solvent and solute may be related to their mass (M) and molecular weight (A) by the following equations: $n_w = M_w/A_w$ and $N_s = M_s/A_s$. Given non-dissociatable species, the VPO readings, given in ideal mmolal, may be related to the vapor pressure of the solution by the following equation (Fullerton et al., 1993):

$$P_1 = \frac{1000g}{18.02g/mole} \div \left(\frac{reading}{1000} + \frac{1000g}{18.02g/mole} \right) \times P_1^\circ \quad (15)$$

This allows one to determine the vapor pressure of the sample solution that is under investigation. Once the vapor pressure of the solution is known, it is possible to calculate the chemical potential of water within the system by the following:

$$\mu_W = RT \ln \frac{P}{P^\circ} \quad (16)$$

The osmotic pressure of the solution may then be calculated according to the following:

$$\Pi = \frac{\mu_W}{\overline{V}} \quad (17)$$

where V is the molar volume of water. This allows one to calculate the osmotic pressure of a solution given readings from the VPO.

Appendix II

Sample Calculation for the Difference in the Regression Coefficients for Two Lines.

The null hypothesis, H^0, is that there is no significant difference between the regression equations for data:

The data points are as follows:

x_1, y_1	x_2, y_2
0,0	0,0
1,1	1,2
2,2	2,4
3,3	3,6
4,4	4,8
5,5	5,10

Summed values required for the calculations are as follows:

$$\sum x_1^2 = 55, \qquad \sum x_2^2 = 55$$

$$\sum x_1 y_1 = 55, \qquad \sum x_2 y_2 = 110$$

$$\sum y_1^2 = 55, \qquad \sum y_2^2 = 220$$

The next step is to calculate the slopes (b) for both lines:

$$b_1 = \frac{\sum x_1 y_1}{\sum x_1^2}, \qquad b_2 = \frac{\sum x_2 y_2}{\sum x_2^2}$$

$$= \frac{55}{55}, \qquad = \frac{110}{55}$$

$$= 1 \qquad = 2$$

next calculate the residual sum of squares (SS) for both samples:

$$\text{residual SS}_1 = \sum y_1^2 - \frac{\left(\sum x_1 y_1\right)^2}{\left(\sum y_1^2\right)}, \text{residual SS}_2 = \sum y_2^2 - \frac{\left(\sum x_2 y_2\right)^2}{\left(\sum y_2^2\right)}$$

$$= 55 - \frac{(55)^2}{55} \qquad = 220 - \frac{(110)^2}{220}$$

$$= 0 \qquad = 165$$

Next, calculate the pooled residual mean square with degrees of freedom = n-2:

$$\left(s_{Y*X}^2\right)_p = \frac{(\text{residual SS})_1 + (\text{residual SS})_2}{(\text{residual DF})_1 + (\text{residual DF})_2}$$

$$= \frac{0+165}{4+4}$$

$$= 20.625$$

Next, calculate the standard error of the difference between regression coefficients:

$$s_{b_1 - b_2} = \sqrt{\frac{\left(s_{Y*X}^2\right)_p}{\left(\sum x^2\right)_1} + \frac{\left(s_{Y*X}^2\right)_p}{\left(\sum x^2\right)_2}}$$

$$= \sqrt{\frac{20.625}{55} + \frac{20.625}{55}}$$

$$= 0.8660254$$

Next, calculate *t*:

$$t = \frac{b_1 - b_2}{s_{b_1 - b_2}}$$

$$= \frac{1-2}{.8660254}$$

$$= 1.1547005$$

The degrees of freedom (v) from the *t*-tables equal:

$$v = n_1 + n_2 - 4$$
$$= 6 + 6 - 4$$
$$= 8$$

For a two tailed test of significance at the 0.05 probability level, the critical *t*-value is $|t_{(n=8,\ p=0.05)}|=1.943$. Since our calculated *t*-value is not less than or equal to the critical t-value, we cannot reject H^0. Therefore there is no significant difference between the regression coefficients between the regression lines observed for the data at the 0.05 level of significance.

Appendix Table I.

Calculated values of the amount of water per PEG (g/g).

wt%	PEG 20 000	PEG 10 000	PEG 8000	PEG 6000	PEG 3000	PEG 2000
15	0.62	1.15	1.13	1.14		
20		1.08	1.01	1.13	1.08	1.25
25		1.01	1.08	1.09	1.00	1.08
30			0.94	1.03	0.86	1.00
35			0.77	0.91	0.80	0.90

wt%	PEG 1500	PEG 1000	PEG 600	PEG 400	PEG 300
5				1.53	
10				1.40	1.69
15	1.15	1.28	1.49	1.28	1.47
20	1.10	1.39	1.29	1.18	1.32
25	1.01	1.07	1.10	1.08	1.23
30	0.94	0.98	0.94	0.99	1.10
35	0.85	0.91	0.80	0.89	0.98

Appendix Table II.

Calculated values of the amount of water per PEG (mole/mole).

wt%	PEG 20 000	PEG 10 000	PEG 8000	PEG 6000	PEG 3000	PEG 2000
5						
10					207.4	106.1
15	690.3	637.2	502.0	380.0	165.8	97.8
20		598.4	448.5	375.3	179.9	139.2
25		558.2	478.6	361.7	165.7	120.2
30			416.3	342.3	143.6	110.6
35			341.7	302.6	133.7	99.6

wt%	PEG 1500	PEG 1000	PEG 600	PEG 400	PEG 300
5			45.7	34.0	
10		102.1	32.3	31.1	28.2
15	95.6	71.3	49.5	28.5	24.5
20	91.8	77.2	42.9	26.3	21.9
25	84.3	59.6	36.6	24.0	20.4
30	78.3	54.5	31.3	22.0	18.3
35	71.1	50.6	26.7	19.9	16.2

Appendix Table III

Measured Osmolalities of Solutions of Different Molecular Weight PEGs in Double Distilled Water.

wt%	PEG 20 000	SEM 20 000	PEG 10 000	SEM 10 000	PEG 8000	SEM 8000
2.5			34	4.47×10^{-1}	33	3.54×10^{-1}
5.0	41	8.16×10^{-1}	45	2.24×10^{-1}	37	5.70×10^{-1}
10.0	57	1.28	57	4.18×10^{-1}	55	0.5
15.0			109	6.52×10^{-1}	118	9.34×10^{-1}
20.0	190	2.13	218	1.34	229	4.18×10^{-1}
25.0			383	2.24×10^{-1}	391	1.15
30.0	659	14.46	670	1.04	653	8.37×10^{-1}
35.0			1046	6.96	1050	2.84

wt%	PEG 6000	SEM 6000	PEG 3000	SEM 3000	PEG 2000	SEM 2000
2.5	41	1.10	30	2.74×10^{-1}	51	9.7×10^{-1}
5.0	47	4.47×10^{-1}	39	4.18×10^{-1}	62	9.7×10^{-1}
10.0	66	5.48×10^{-1}	70	6.52×10^{-1}	94	1.23
15.0	126	6.52×10^{-1}	157	2.74×10^{-1}	196	1.60
20.0	239	1.30	270	5.70×10^{-1}	314	3.69
25.0	402	1.00	456	4.18×10^{-1}	517	1.30
30.0	656	1.04	731	2.43	806	11.42
35.0	1028	9.08×10^{-1}	1114	9.08×10^{-1}	1194	2.69
40.0					1759	28.66

wt%	PEG 1500	SEM 1500	PEG 1000	SEM 1000	PEG 600	SEM 600
2.5			50	3.16×10^{-1}	64	6.78×10^{-1}
5.0	59	1.29	72	1.14	103	4.00×10^{-1}
10.0	127	1.45	134	2.11	246	6.00×10^{-1}
15.0	222	1.26	282	8.37×10^{-1}	395	4.01
20.0	365	1.33	434	1.28	617	2.26
25.0	581	2.58	696	2.03	915	3.23
30.0	879	3.43	1024	3.14	1310	3.57
35.0	1283	1.11	1458	6.96	1835	5.10

wt%	PEG 400	SEM 400	PEG 300	SEM 300
2.5	77	3.16×10^{-1}	90	7.75×10^{-1}
5.0	138	1.63	210	7.07×10^{-1}
10.0	328	1.79	388	6.00×10^{-1}
15.0	537	9.49×10^{-1}	653	1.05
20.0	830	2.27	993	5.10×10^{-1}
25.0	1204	4.08	1433	2.64
30.0	1711	14.84	1963	2.20

-columns headed PEG--- give measured concentrations from the vapour pressure osmometer given in milliosmolal.

-columns headed SEM--- give the standard errors of the means for five measurements on the vapour pressure osmometer.

-PEG solutions were prepared by dissolving PEG in various weight percent concentrations (weight PEG/total solution weight) in double distilled water.

Appendix Table IV.

Osmotic pressures obtained from the vapour pressure osmometer.

wt%	PEG 20 000	SEM 20 000	PEG 10 000	SEM 10 000	PEG 8000	SEM 8000
2.5			5.947	14.534 x 10^{-3}	5.929	3.520x 10^{-3}
5.0	6.023	8.763x 10^{-3}	6.066	1.743x 10^{-3}	5.984	4.990x 10^{-3}
10.0	6.165	9.646x 10^{-3}	6.165	2.607x 10^{-3}	6.151	2.986x 10^{-3}
15.0			6.448	2.131x 10^{-3}	6.483	2.592x 10^{-3}
20.0	6.690	4.889x 10^{-3}	6.750	2.178 x10^{-3}	6.771	6.012x 10^{-4}
25.0			6.996	2.075x 10^{-4}	7.005	9.672x 10^{-4}
30.0	7.232	9.626x 10^{-3}	7.240	5.508x 10^{-4}	7.228	4.225x 10^{-4}
35.0			7.434	2.378x 10^{-3}	7.436	8.975x 10^{-4}

wt%	PEG 6000	SEM 6000	PEG 3000	SEM 3000	PEG 2000	SEM 2000
2.5	6.019	9.100x 10^{-3}	5.894	2.949x 10^{-3}	6.120	6.586x 10^{-3}
5.0	6.079	3.130x 10^{-3}	6.004	3.517x 10^{-3}	6.205	5.326x 10^{-3}
10.0	6.232	2.726x 10^{-3}	6.257	3.042x 10^{-3}	6.385	4.760x 10^{-3}
15.0	6.511	1.706x 10^{-3}	6.606	5.753x 10^{-3}	6.705	2.803x 10^{-3}
20.0	6.789	1.797x 10^{-3}	6.842	6.963x 10^{-4}	6.909	4.038x 10^{-3}
25.0	7.017	8.185x 10^{-4}	7.071	3.025x 10^{-4}	7.126	8.700x 10^{-4}
30.0	7.230	5.232x 10^{-4}	7.278	1.089x 10^{-3}	7.320	4.882x 10^{-3}
35.0	7.427	2.928x 10^{-4}	7.462	2.704x 10^{-4}	7.492	7.844x 10^{-4}
40.0					7.664	5.674x 10^{-3}

wt%	PEG 1500	SEM 1500	PEG 1000	SEM 1000	PEG 600	SEM 600
2.5			6.110	2.323 x 10^{-3}	6.214	3.926x 10^{-3}
5.0	6.184	7.979x 10^{-3}	6.268	5.878x 10^{-3}	6.426	1.426x 10^{-3}
10.0	6.515	4.129x 10^{-3}	6.539	5.704x 10^{-3}	6.802	8.954x 10^{-4}

15.0	6.758	2.098x 10^{-3}	6.862	1.089x 10^{-3}	7.008	3.730x 10^{-3}
20.0	6.975	1.336x 10^{-3}	7.050	1.088x 10^{-3}	7.203	1.347x 10^{-3}
25.0	7.177	1.638x 10^{-3}	7.260	1.079x 10^{-3}	7.376	1.302x 10^{-3}
30.0	7.358	1.444x 10^{-3}	7.425	1.134x 10^{-3}	7.533	1.012x 10^{-3}
35.0	7.524	3.225x 10^{-4}	7.580	1.765x 10^{-3}	7.682	1.038x 10^{-3}

wt%	PEG 400	SEM 400	PEG 300	SEM 300
2.5	6.297	1.629x 10^{-3}	6.365	4.137x 10^{-3}
5.0	6.549	4.809x 10^{-3}	6.734	1.638x 10^{-3}
10.0	6.928	2.303x 10^{-3}	7.002	7.545x 10^{-4}
15.0	7.142	5.467x 10^{-4}	7.228	7.8597x 10^{-4}
20.0	7.333	9.201x 10^{-4}	7.412	2.5146x 10^{-4}
25.0	7.495	1.210x 10^{-3}	7.573	9.0664x 10^{-4}
30.0	7.651	3.410x 10^{-3}	7.711	5.5382x 10^{-4}

-Readings are given as log osmotic pressure values in dynes/cm^2.
-Values are averages of five readings on the osmometer.

Appendix Table V.

Ratios of the concentration of glucose with the addition of various molecular weight PEG to the concentration of glucose at zero pressure.

WT%	PEG 20 000	PEG 10 000	PEG 8000	PEG 6000	PEG 3000	PEG 2000
2.5		0.89	0.91	0.88	0.93	0.82
5	0.93	0.92	0.95	0.93	0.99	0.89
10	1.07	1.10	1.13	1.10	1.16	1.12
15		1.25	1.25	1.25	1.21	1.18
20		1.37	1.34	1.39	1.37	1.46
25		1.50	1.56	1.57	1.50	1.56
30			1.67	1.79	1.59	1.74
35			1.71	1.96	1.76	1.94

WT%	PEG 1500	PEG 1000	PEG 600	PEG 400	PEG 300
2.5		0.93	0.96	1.01	1.03
5	1.02	1.04	1.08	1.09	1.05
10	1.11	1.26	1.12	1.18	1.23
15	1.25	1.29	1.36	1.29	1.35
20	1.38	1.53	1.48	1.42	1.49
25	1.51	1.56	1.58	1.56	1.69
30	1.68	1.73	1.68	1.74	1.89
35	1.85	1.97	1.76	1.93	2.11

-Measurements were made on the vapour pressure osmometer.

Appendix Table VI.

Osmotic pressures for PEG MW 1500, 1000, 400, and 300 as measured by secondary osmometry with the phospholipid SOPC.

wt% PEG	log P 1500	log P 1000	log P 400	log P 300
5	5.92	6.07	5.91	6.08
10	6.49	6.61	6.64	6.60
15	6.78	6.82	6.85	7.00
20	6.89	7.08	7.20	7.11
25	7.27	7.34	7.43	7.34
30	7.41	7.46	7.49	7.71
35	7.38	7.55	7.61	7.88
40	7.60	7.65	8.00	8.01
45	7.63	7.79	8.16	8.28
50	7.83	7.89	8.25	8.31
55	7.59	7.93	8.32	8.43
60	8.03	7.95	8.44	8.52

-Values given as log osmotic pressure in dynes/cm^2.
-The multilamellar x-ray diffraction patterns of stearyloleoylphosphatidylcholine (SOPC) were measured in 2 mmolar TES (Sigma) buffer (pH 7) or PEG/TES solutions at 25 °C.

Appendix Table VII.

Glucose equilibrium dissociation constant (GEDC) values measured in the presence of all the different MW PEGs.

wt %	PEG 20000-1	PEG 20000-2	PEG 10000-1	PEG 10000-2	PEG 10000-3	PEG 8000-1
5	0.126	0.113	0.144	0.128	0.149	0.159
10	0.078	0.077	0.110	0.112	0.114	0.125
15	0.089	0.069	0.075	0.075	0.063	0.055
20			0.051	0.054	0.060	0.037
25			0.054	0.054	0.049	0.033
30						0.024

wt %	PEG 8000-2	PEG 8000-3	PEG 6000-1	PEG 6000-2	PEG 6000-3	PEG 4000-1
5	0.112		0.123	0.138	0.137	0.125
10	0.087	0.078	0.098	0.095	0.092	0.107
15	0.081	0.074	0.075	0.072	0.064	0.082
20	0.063	0.042	0.054	0.057	0.044	0.039
25	0.039	0.024	0.040	0.044	0.043	0.039
30	0.024		0.020	0.020	0.023	0.027

wt %	PEG 4000-2	PEG 4000-3	PEG 4000-4	PEG 3000-1	PEG 3000-2	PEG 3000-3
5	0.125	0.124	0.105	0.118	0.129	0.137
10	0.115	0.077		0.092	0.099	0.092061
15	0.072			0.070	0.065	0.064
20	0.045	0.057	0.044	0.043	0.045	0.044
25	0.039	0.055	0.032	0.035	0.034	0.033
30	0.029	0.025	0.026	0.028	0.019	0.026

wt %	PEG 2000-1	PEG 2000-2	PEG 2000-3	PEG 2000-4	PEG 1500-1	PEG 1500-2
5	0.126	0.145	0.127	0.157	0.123	0.126
10	0.095	0.090	0.101	0.090	0.086	0.091
15	0.062	0.064	0.068	0.076	0.061	0.045
20	0.043	0.042	0.047	0.043	0.041	0.041
25	0.029	0.028	0.033	0.036	0.026	0.028
30	0.019	0.018	0.024	0.023	0.022	0.024

wt %	PEG 1500-3	PEG 1500-4	PEG 1500-5	PEG 1000-1	PEG 1000-2	PEG 600-1
5	0.130	0.130	0.120	0.127	0.122	0.133
10	0.103	0.089	0.102	0.092	0.100	0.098
15	0.075	0.066	0.073	0.068	0.075	0.057
20	0.040	0.045	0.047	0.047	0.043	0.052
25	0.026	0.039	0.028	0.029	0.030	0.031
30	0.021	0.020	0.022	0.021	0.020	0.023

wt %	PEG 600-2	PEG 400-1	PEG 400-2	PEG 300-1	PEG 300-2
5	0.133	0.144	0.135	0.149	0.158
10	0.103	0.094	0.096	0.110	0.094
15	0.072	0.069	0.082	0.073	0.064
20	0.055	0.048	0.056	0.052	0.044
25	0.031	0.033	0.036	0.035	0.045
30	0.024	0.023	0.023	0.028	0.025

-GEDC values measured in mmolal.
-the numbers after the MW of the PEGs indicate separate experiments.
-GEDC values were calculated on a Macintosh LC 475 using the program Multifit (version 2) from changes in the normalized fluorescence of hexokinase upon binding to glucose.

Appendix Table VIII.

Linear regression coefficients describing the relationships between the log glucose affinity ratios (mmolar) and osmotic pressure (dynes/cm^2*10^7).

PEG MW	Correlation Coefficient	Number of Observations
10 000	0.866	18
8 000	0.761	19
6 000	0.836	21
3 000	0.958	21
2 000	0.867	28
1 500	0.857	35
1 000	0.981	14
600	0.971	14
400	0.969	14
300	0.900	14

Appendix Table IX.

The calculated t-values for determining the differences slopes of the log affinity data.

PEG MW	PEG MW 300	PEG MW 400	PEG MW 600	PEG MW 1000
PEG 300	-	t=1.5567 n=28	t=3.7361** n=28	t=4.9815** n=28
PEG MW 400	t=1.5567 n=28	-	t=2.9027* n=28	t=6.283** n=28
PEG MW 600	t=3.7361** n=28	t=2.9027* n=28	-	t=2.7789** n=28
PEG MW 1000	t=4.9815** n=28	t=6.2283** n=28	t=2.7789* n=28	-

* testing is significant at the 0.05 level of significance.
** testing is significant at the 0.005 level of significance.

Appendix Table X.

The calculated changes in the numbers of polymer inaccessible water molecules upon the binding of glucose to hexokinase.

Pressure PEG 10 000	ΔN_w PEG 10 000	Pressure PEG 8000	ΔN_w PEG 8000	Pressure PEG 6000	ΔN_w PEG 6000
0.12	288.27	0.10	271.14	0.12	203.95
0.15	264.92	0.14	246.40	0.17	162.34
0.28	208.59	0.30	173.56	0.32	113.08
0.56	115.03	0.59	92.60	0.62	77.70
		1.01	56.44	1.04	65.21
		1.69	34.69	1.70	48.57

Pressure PEG 3000	ΔN_w PEG 3000	Pressure PEG 2000	ΔN_w PEG 2000	Pressure PEG 1500	ΔN_w PEG 1500
0.13	188.57	0.16	190.87	0.15	181.41
0.31	114.63	0.24	175.74	0.33	128.44
0.57	95.68	0.51	115.64	0.57	101.89
0.94	75.46	0.81	69.06	0.94	73.08
1.46	66.87	1.34	58.79	1.50	51.64
2.22	52.76	2.09	42.77	2.28	39.02

Pressure PEG 1000	ΔN_w PEG 1000	Pressure PEG 600	ΔN_w PEG 600	Pressure PEG 400	ΔN_w PEG 400
0.19	140.59	0.27	88.46	0.36	62.53
0.35	113.67	0.63	69.30	0.85	42.18
0.73	89.47	1.02	55.42	1.39	38.24
1.12	79.24	1.60	48.70	2.15	31.03
1.80	61.86	2.38	42.94	3.14	27.01
2.66	42.43	3.42	34.51	4.48	25.45

Pressure PEG 300	ΔN_w PEG 300
0.54	42.00
1.00	38.68
1.69	30.75
2.58	23.34
3.74	18.40
5.15	14.89

-pressure is given in dynes/cm^2*10^7.